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CHOLESTEROL TEMPLATED POLYMERS: ARE THEY REALLY SPECIFIC BINDING MATERIALS?

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ABSTRACT

Cholesterol Templated Polymers: Are They Really Specific Binding Materials?

Master of Science 2008, Molecular Science Program

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The technique of molecular imprinting creates template specific and selective polymer products with a large assortment of applications. For example, molecularly imprinted polymers (MIPs) may pose as a means to separate undesirable components, such as endocrine disruptors, from the environment. Taking advantage of β-cyclodextrin's ability to form inclusion complexes with a number of guests has led scientists to use them as scaffolds in the synthesis of MIPs. Komiyama's approach of molecularly imprinting cholesterol with β-cyclodextrin was used as a starting point to apply MIPs as potential tools for trapping endocrine disruptors. This study presents results on the re-binding of cholesterol to a cholesterol-templated MIP and to a non-MIP (NMIP), as well as the binding of a series of structurally unrelated compounds to the cholesterol-templated MIP and NMIP. The results consistently show that cholesterol-templated MIPs synthesized using Komiyama's method lack specificity and selectivity for their template. This calls into question their efficacy as a tool for trapping endocrine disruptors.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

Prologue

Endocrine disruptors are of current concern as contaminants in drinking water and foods. They are often found at very low concentrations and therefore there is value in developing highly specific and efficient trapping materials as a way to eliminate them. For example, incorporating these trapping materials in order to remove trace amounts of endocrine disruptors from household water input would be invaluable. One approach in order to achieve this is to take advantage of the very high binding specificity and selectivity afforded through molecular recognition. There is a proposed technique that fabricates molecular recognition into materials and it is called molecular imprinting. The initial impetus behind this study was to examine the claim that molecularly imprinted polymers designed using cyclodextrins are useful for specific and efficient trapping of model endocrine disruptors. The objective was to molecularly imprint cholesterol, which is structurally related to some endocrine disruptors, using a well documented method and to test the specificity and selectivity of the resulting molecularly imprinted polymers. Once this was achieved, the next objective was to extend this methodology to molecularly imprinted polymers designed to trap actual endocrine disruptors.

1.1 Endocrine Disruptors

Endocrine disruptors (EDs) are a group of structurally variable molecules that are associated with a number of unfavourable consequences seen in invertebrates and vertebrates.[1,2] They are defined by the United States Environmental Protection Agency as "exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis".[3] More specifically, EDs "mimic or antagonize the effects of endogenous hormones, such as estrogens and androgens, or disrupt synthesis and metabolism of endogenous hormones and hormone receptors".[4] At the biochemical level, EDs interfere with normal animal functions in three main ways: mimicking, blocking, or triggering a hormone's response.[3,5]

There are different types of conventional EDs such as: natural substances (i.e., phytoestrogens), pesticides (i.e., DDT), fungicides (i.e., vincozolin), plasticizers (i.e., bisphenol A), industrial chemicals (i.e., polychlorinated biphenyls), and metals (i.e., mercury).[3,4,5] There is also a new and emerging group of EDs including surfactants, human and veterinary drugs, fragrances, and antiseptics.[6] Depending on the geographical region, EDs are found throughout the environment in different amounts.[1] Although the relative quantities are very small, EDs are said to negatively impact humans, wildlife, fish, and their offspring.[1] Some of the adverse effects associated with EDs include: altered reproductive fitness, such as reproductive problems in females and males, birth defects, cancer, and compromised immune functioning.[5] The fact that most of them are lipophilic promotes their bioaccumulation and biomagnification in the environment.[1] Many of the reports are based on aquatic organisms and the species that feed on them because their environments tend to be the sink for the waste that contains these EDs.[2] However it is feasible to correlate the effects seen in aquatic animals to that of humans.[4]

EDs find their way into the environment from the wastewater of industrial and domestic outlets, sewage treatment plant effluents, and runoff from agriculture. They then go directly into rivers, lakes, and oceans before any treatment is applied.[1,4]

There are a number of problems coupled with effectively removing EDs from the environment. For example, conventional wastewater treatment methods are not adequate to remove most if not all of the EDs.[5] Other methods such as granular activated carbon, powder activated carbon, coagulation/filtration, and lime softening are expensive and require a skilled operator in order to carry out the process.[5] Thus, it would be highly valuable to take the time and put in the effort to generate a method of ED-removal that is effective, specific, selective, inexpensive, and easy to use and/or incorporate into today's water treatment systems.

1.2 Molecular Imprinting

Molecular recognition is a key step in many biochemical processes. Membrane interactions, enzymatic catalysis, drug interactions, cell-signalling, immune response, and DNA replication are some common examples of molecular recognition. Because of its central role in biochemistry, molecular recognition has been intensely studied for many decades.[7]

Lehn defined molecular recognition as "the energy and the information involved in the binding and selection of substrate(s) by a given receptor molecule and may also involve a specific function".[7] The

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analogy most often associated with molecular recognition is the lock-and-key (Figure 1.1). This analogy emphasises the notion and importance of specificity and selectivity in order to achieve molecular recognition.

While the original impetus for its study came from biochemistry, it became apparent over time that molecular recognition could also be designed into synthetic systems. The method known as molecular imprinting of polymers is an example of an attempt to create a synthetic molecular recognition system providing both specific and selective interactions.[8,9,10,11]

Molecular imprinting is, in essence, the formation of artificial receptor sites in a polymer matrix.[12,13] (Figure 1.2) There are a number of benefits associated with molecular imprinting. As Xu *et. al* put it: "Molecularly imprinted polymers have physical robustness, high strength, resistance to elevated temperature and pressures, and inertness to acids, bases, metal ions and organic solvents as well as the low cost and ease for preparation".[14] But the key advantage to molecular imprinting is the potential for highly specific and strong binding of a particular molecule to the imprinted polymer. These features have led to attempted applications in areas such as purification, separation, sensors, catalysis, and drug delivery.[9,13,15-18]

There are four components typically used to achieve molecular imprinting: a template, functional monomer(s), crosslinker, and (usually, but not always) a solvent. The relative quantities of these components are, crosslinker>functional monomer(s)>template.[19]

The template, which is sometimes referred to as the guest, is the molecule of interest or, to use a term from enzymology, the substrate. The size, shape, binding complementarity, and orientation of the template will be imprinted within the polymer.[20] Here, binding complementarity refers both to spatial orientation and to the complementary positioning of functional groups which result in the interaction of the template with the polymer forming components (i.e., functional monomer and crosslinker).[13,19,21]

The functional monomer interacts with the template to form a pre-organized assembly.[12] In order to ensure the formation of the imprint a molar ratio of 1:4 or greater of the template to functional monomer is typically used.[19] The most commonly used functional monomer in molecular imprinting is methyl methacrylate (See Figure 1.3 for a more complete list).[20] Depending on the molecular details of the template, there may be more than one type of functional monomer necessary to interact with the functional groups of the template. Furthermore, the functional monomer must have two critical components: one to interact with the template to form a pre-organized structure and the other to react with the crosslinker to achieve polymerization.[9]

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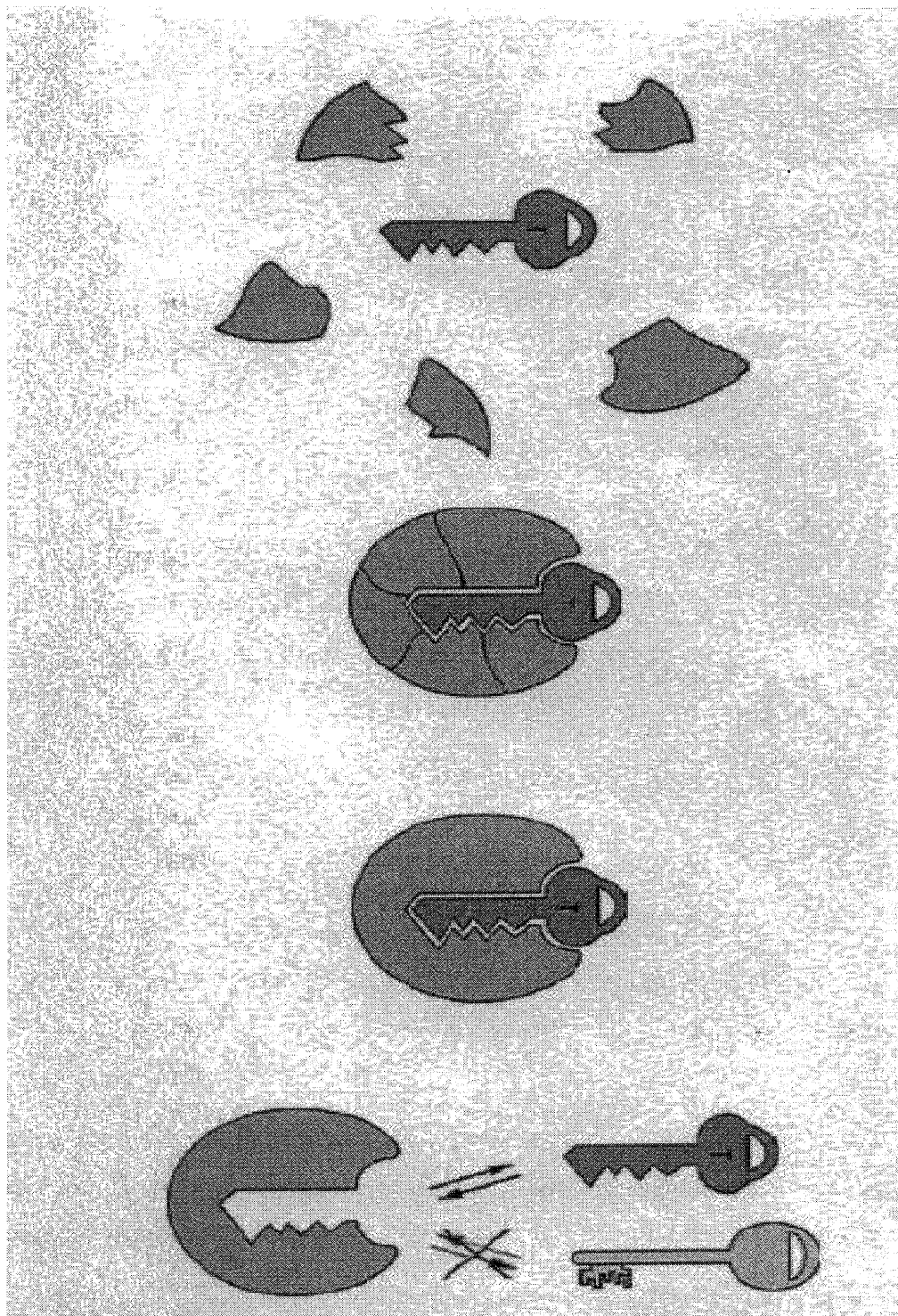
Figure 1.1 Fisher's lock-and-key metaphor (Reproduced from Yan *et. al*, 2005).[13]

The crosslinker covalently binds to the functional monomers, essentially locking everything into place and completing the polymerization. The consensus is to use equal to or greater than an 80% excess of crosslinker compared to the functional monomer for the best results.[19,22] The most commonly used crosslinkers are ethylene glycol dimethacrylate and divinylbenzene (See Figure 1.4 for a more complete list).[20,23] The amount of crosslinker used determines the morphology of the final product, usually ranging from a gel-like consistency to a hard material.[19] Determining the amount of crosslinker to use is a crucial step because adding too much may create a very rigid polymer and adding too little may create too flexible a polymer.

In most cases molecular imprinting takes place within organic solvents or aqueous solutions. Sometimes a solvent is not needed because the functional monomer(s) and crosslinker are already in the liquid phase. There are three main purposes for using a solvent. The first is to combine all the components in a single phase. The second is to create pores within the polymer matrix, and the third is to make it possible for the template and functional monomer to interact.[19]

Once polymerization is complete and the template has been molecularly imprinted, the template is removed by various methods and the synthetic binding sites are retained within the polymer matrix.

There are three approaches to molecular imprinting: the non-covalent method, the covalent method, and the semi-covalent method.

The non-covalent method was developed by Mosbach, and involves non-covalent interactions between the template and functional monomer. The types of non-covalent interactions include electrostatic, hydrogen bonding, hydrophobic interactions, and Van der Waals forces.[12,13,23] The subsequent re-binding of the template by the artificial binding sites is also through non-covalent interactions.[13]

The covalent method of molecular imprinting was developed by Wulff, and requires that the template and functional monomer interact via covalent bonds. The only way to reverse the bonds formed by the covalent molecular imprinting method is through a cleavage step. The subsequent re-binding of the template also involves formation of covalent bonds.[13]

Finally, the semi-covalent method involves the formation of covalent bonds between the template and functional monomer, which are cleaved after molecular imprinting is completed. Alternatively, the re-binding of the template is through non-covalent interactions.[9,13]

Assembly

Polymerization

Extraction of T

Rebinding of T

T   M   L   MIP   MIP
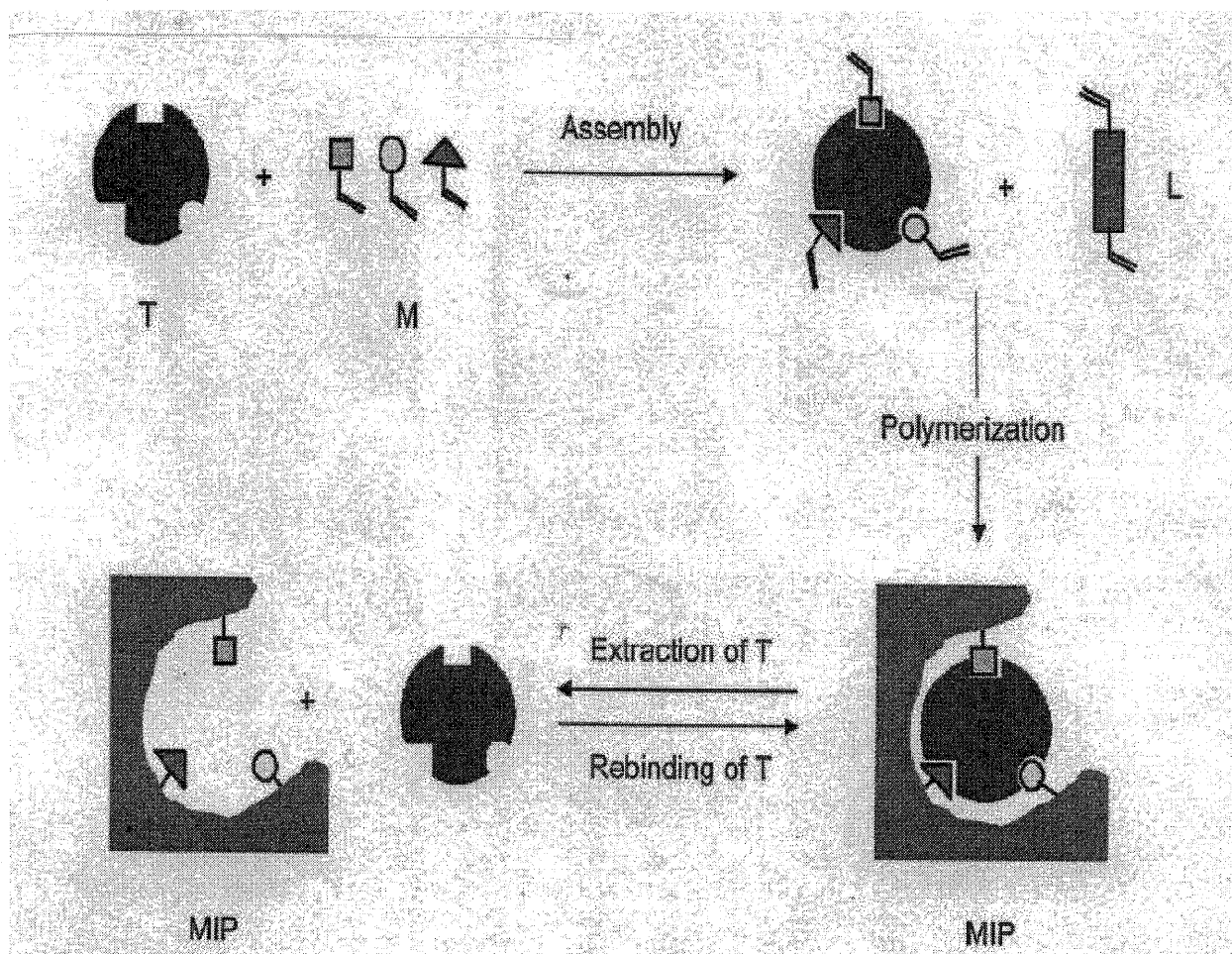
Where T=template, M=functional monomer, and L=crosslinker

Figure 1.2 Schematic Illustration of Molecular Imprinting (Reproduced from Yan *et. al*, 2005).[13]

Methacrylic acid — Trifluoro-methacrylic acid — Acrylamido-(2-methyl)-propane sulfonic acid — 4-Vinylbenzoic acid — Itaconic acid

4-Vinylpyridine — 4-Vinylbenzyl-trimethyl-ammonium chloride — 4(5)-Vinylimidazole — 1-Vinylimidazole — 4-Vinylbenzamidine

Hydroxyethylmethacrylate — Styrene — Acrylamide — Vinylpyrrolidone — Methylmethacrylate — Acrylonitrile

4-Vinylbenzyl-iminodiacetic acid — Methyl-a-D-glucopyranoside-6-acrylate — Fluorescent reporter monomer

Figure 1.3 Commonly used Functional Monomers for Molecular Imprinting (Reproduced from Yan *et. al*, 2005).[13]

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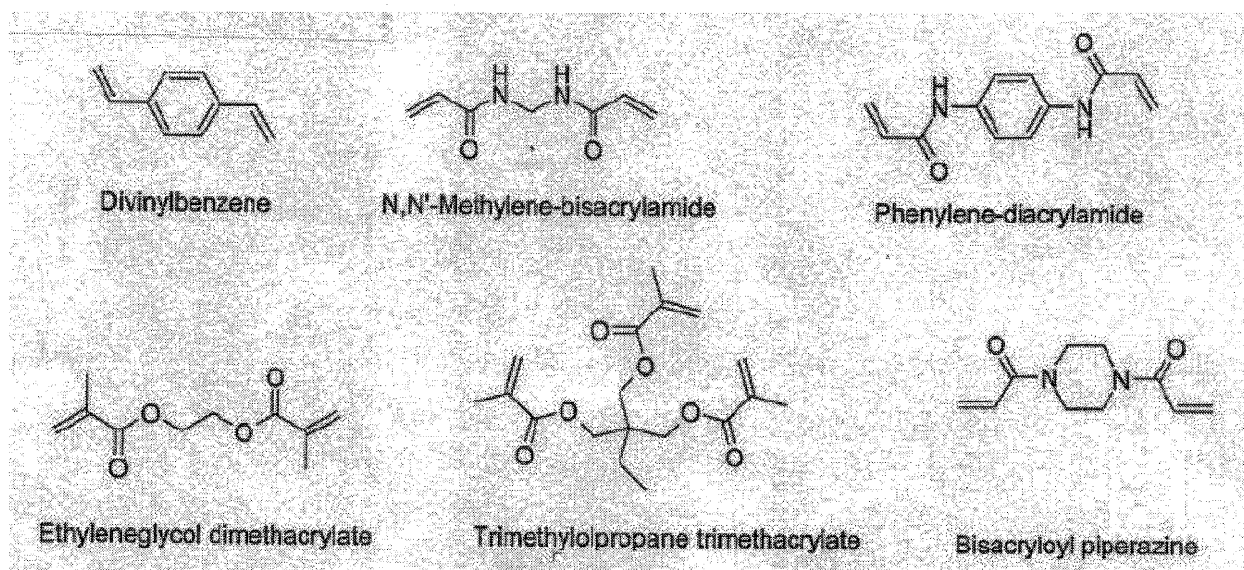
Figure 1.4 Commonly used Crosslinkers for Molecular Imprinting (Reproduced from Yan *et. al,* 2005).[13]

1.3 Cyclodextrins

Cyclodextrins (CDs) are a class of cyclic oligosaccharides, which consist of several linked glucose units. They are obtained from corn starch by the action of the enzyme cyclodextrin glycosyltransferase.[24] CDs are named according to the number of glucose units present and therefore the size of the molecule. For example, α–CD has six glucose units, β–CD has seven (Figure 1.5), and γ–CD has eight. Each glucose unit is joined to the next by a α–1, 4-linkage.[25] The resulting structure of the CD molecules is a torus shaped cavity.[26] There are two rims on a CD molecule, one of them narrower than the other, but both are lined with hydroxyl groups. The hydroxyl groups on the narrower face are referred to as the primary hydroxyl groups, and the hydroxyls situated on the other face are referred to as the secondary hydroxyl groups.[26] These hydroxyl groups give CDs their hydrophilic character by forming hydrogen bonds with water molecules. The hydroxyl groups are also reactive and are the site for derivatization and polymerization of CDs. On the other hand, the CD cavity interior is composed of carbon and oxygen atoms which make it hydrophobic.[26] CDs are, thus, amphiphilic.

There has been a tremendous amount of work done on the ability of CDs to form inclusion complexes with a variety of guests. Their capability to form complexes is mostly due to their hydrophobic core. For example, CDs are known to form inclusion complexes with the hydrophobic molecule cholesterol. In fact, because cholesterol is a large molecule it forms an inclusion complex with β-CD at a ratio of 1:3.[27] The type of interaction between the CD cavity and a guest is through non-covalent interactions, and is a "dynamic process whereby the guest molecule continuously associates and dissociates from the host CD".[28]

CDs have been shown to have numerous applications, mostly based on their inclusion complex formation property. CDs have been used in separation applications, drug delivery systems, sensor applications, and as stabilizers and solublizers.[29-32]

1.4 Molecular Imprinting Using Cyclodextrins

As previously mentioned CDs act as hosts that form inclusion complexes with a guest molecule. Therefore, CDs are able to form the necessary pre-organized structures with a template molecule in order to ensure that the custom-made receptors are properly formed before the crosslinker is added during molecular imprinting.[30] Although the individual interactions between the CD and template are quite weak, very high selectivity and binding is accomplished when a number of them work altogether.[23] CDs

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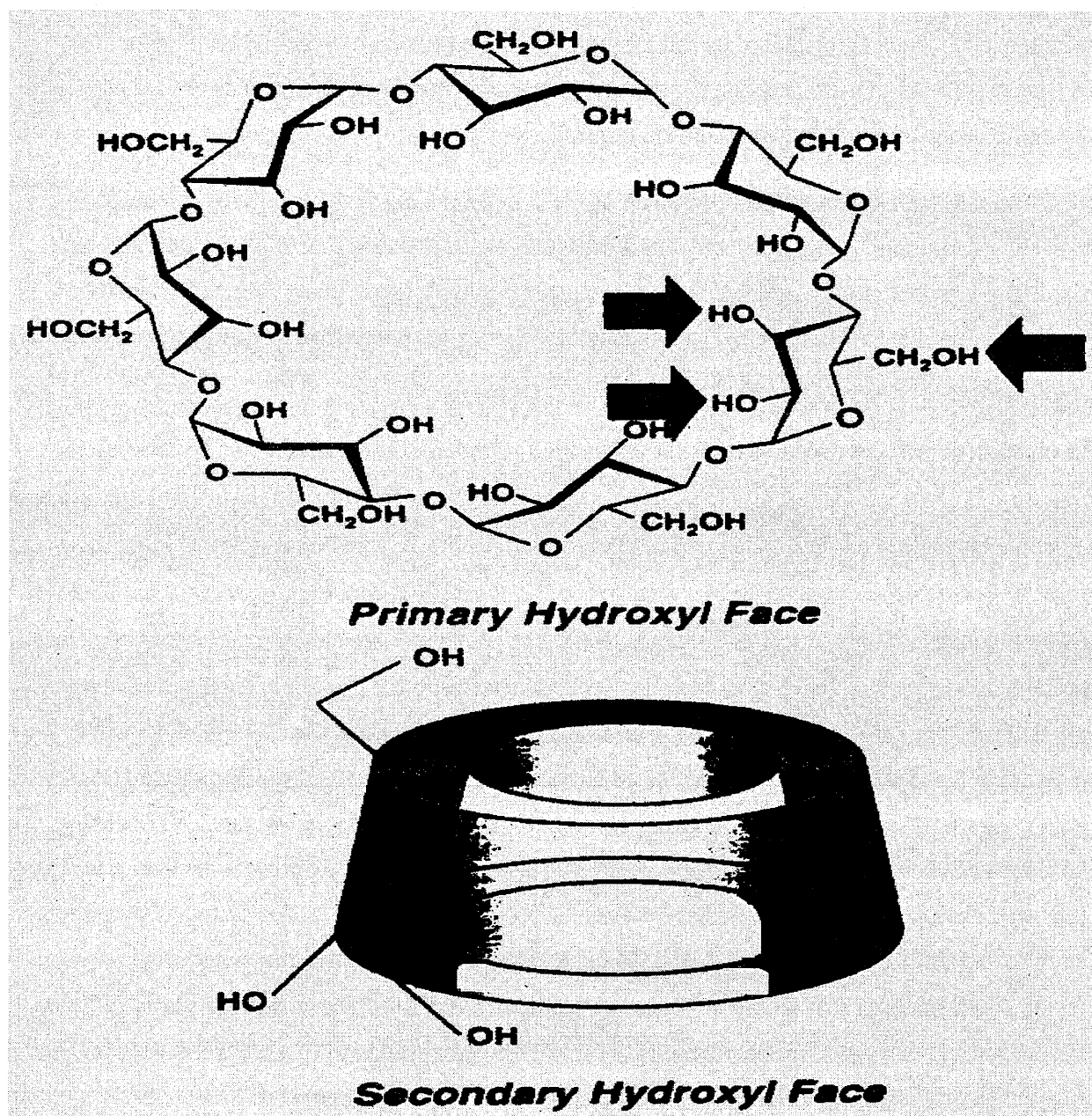
Figure 1.5 Structure of β-Cyclodextrin (Reproduced from Hirayama *et. al*, 1999).[31]

can also bind larger guests, such as steroids which makes them an attractive choice for molecular imprinting.[33-37] Another benefit of using CDs is the ability to perform all polymer syntheses and re-binding procedures in water, making it more environmentally friendly and applicable to food and health industries due to the lack of organic solvent usage.[36]

Komiyama *et. al*, developed two separate methods of using CDs as the functional monomers in molecular imprinting.[36] The first method takes place in water. CDs are first treated so that they have vinyl groups before pre-organization and molecular imprinting can take place. The second method is in an organic solvent, such as dimethyl sulfoxide (DMSO) and does not require CDs to be derivatized prior to pre-organization and molecular imprinting (Figure 1.6).

In experiments using the second molecular imprinting procedure, Komiyama *et. al*, used cholesterol (Figure 1.7) as the template and β–CD as the functional monomer (Figure 1.5). Since cholesterol is a hydrophobic molecule it forms a complex with the hydrophobic cavity of the β–CD molecule. The solvent used is DMSO which is a polar organic solvent, where both cholesterol and β–CD do bind with a modest binding constant of $550M^{-1}$.[36] When β–CD and cholesterol are dissolved in DMSO, they form a pre-organized structure which is visibly observed by the formation of a precipitate. Since cholesterol is too large to fit in only one β–CD cavity, it was suggested that one end of the cholesterol molecule interacts with the cavity of one CD and the other end interacts with an adjacent CD molecule (Figure 1.8).[36] This CD "dimer" is the pre-organized structure. Both tolylene 2,4-diisocyanate (TDI) and hexamethylene diisocyanate (HMDI) can be used as the crosslinkers. According to the literature, TDI is the better choice of the two crosslinkers because the polymers made with TDI showed a higher re-binding activity than those made with HMDI.[35,36] In either case, the crosslinker reacts with the hydroxyl groups on the β–CD molecules and forms a urethane linkage between them. The crosslinker reacts with the CD's hydroxyls instead of the cholesterol's because the hydroxyls on the CDs are more reactive than those on the cholesterol, and because there is an excess of CD molecules over cholesterol present in the reaction mixture. When polymerization is complete and the template is removed the binding sites for cholesterol are retained within the polymer.[36]

6-mer: α-cyclodextrin
7-mer: β-cyclodextrin
8-mer: γ-cyclodextrin

diisocyanate

TDI =

HMDI =
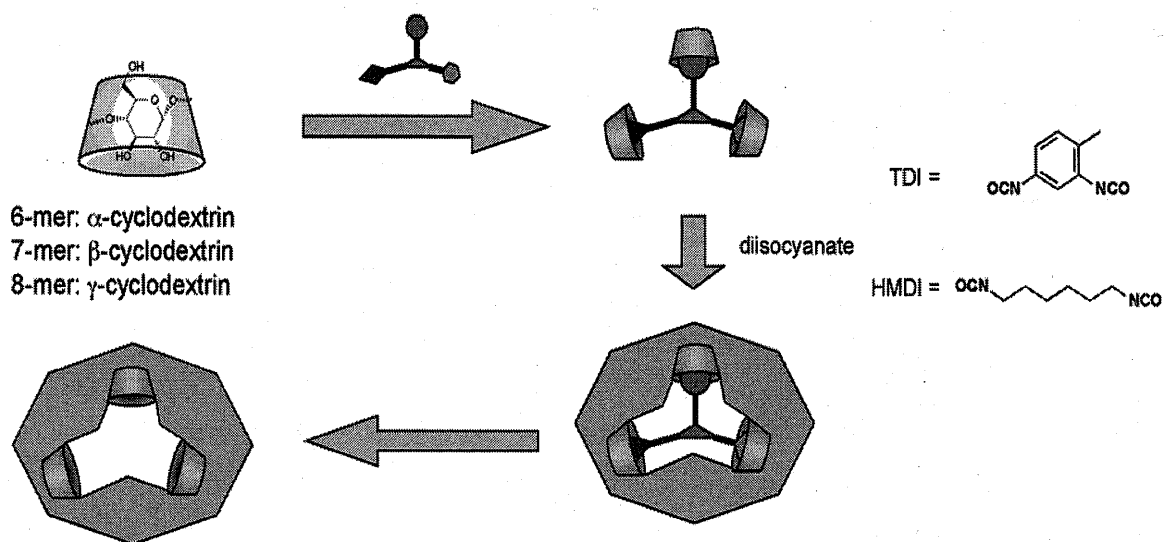
Figure 1.6 Schematic Illustration of Molecular Imprinting in DMSO using Cyclodextrins as the Functional Monomer (Reproduced from Asanuma *et. al,* 2000).[36]

1.5 Cholesterol and Cholesterol Molecularly Imprinted Polymers (MIPs)

Cholesterol (Figure 1.7) is a lipid molecule found in plants, animals, cell membranes, and foods. It plays an especially important role in the structure of eukaryotic cell membranes. It is one of the required building blocks for membrane synthesis and it also regulates the fluidity of the cell membrane itself.[38]

The cholesterol molecule is classified as a sterol or alternatively a steroid alcohol, which is a subclass of the structurally related molecules called steroids. Like all other sterols, cholesterol is hydrophobic and thus poorly soluble in water.

It is well known that excess cholesterol can have adverse health affects on humans. A diet high in cholesterol is associated with high blood pressure, atherosclerosis, and coronary heart disease.[39,40] As a result, there has been research done to remove or lower the cholesterol content from certain foods during manufacturing. Cholesterol binds to β–CD and there have been a number of studies that used β–CD as a way to separate cholesterol from various food products. This method successfully reduced cholesterol content in dairy products, such as whipping cream, egg yolks, and milk.[41,42] There has also been work done applying cholesterol-templated MIPs as a way to remove cholesterol from foods which could potentially remove a greater amount of cholesterol due to the more specific and selective nature of MIPs, compared to using free β–CD. Also, MIPs are more easily recovered from the food manufacturing process than are free β–CDs.

1.6 Objectives of the Study

In this study, cholesterol is used as a model for EDs. There is good evidence from the literature that the MIP approach can be used to trap trace amounts of cholesterol. The objective here was initially to evaluate the re-binding ability of a β–CD cholesterol MIP and then to extend the methodology to steroid-like EDs, since both are structurally related. Based on the preliminary findings, the original plan was altered and the focus shifted to the specificity and selectivity, or lack thereof, of the β–CD cholesterol MIP.

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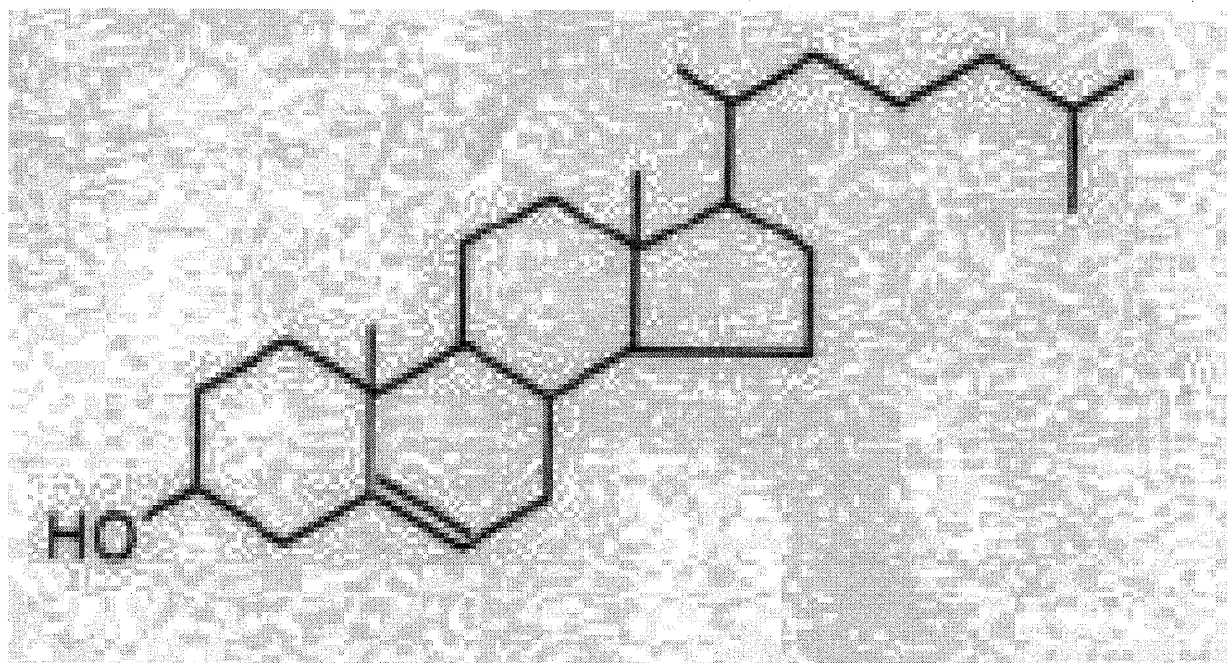
Figure 1.7 Structure of Cholesterol (Reproduced from Heftmann, 1970).[43]
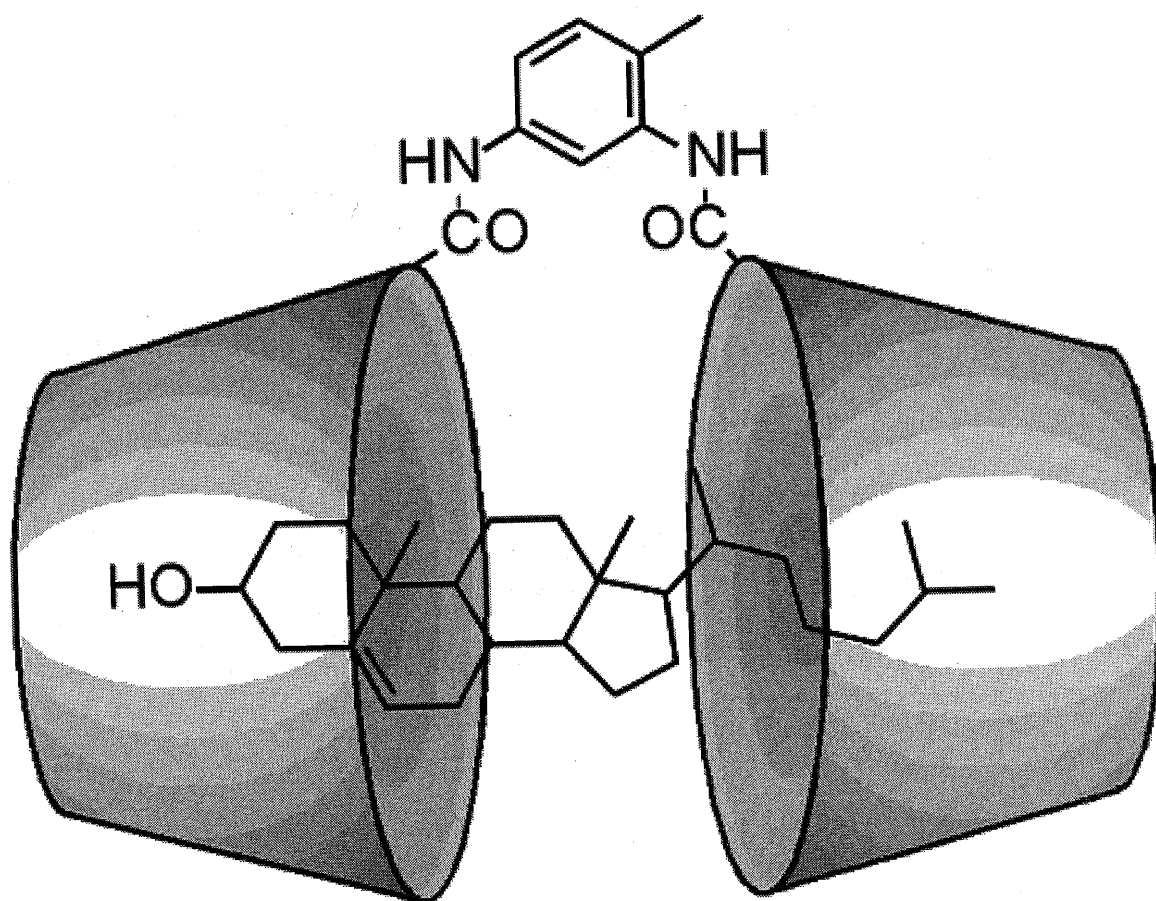
Figure 1.8 Proposed Model of Cholesterol-Imprinted Site when Cyclodextrins are used as the Functional Monomer (Reproduced from Asanuma *et. al,* 2000).[36]

CHAPTER 2

EXPERIMENTAL

2.1 Materials

The following items were used to synthesize the two sets of polymers (molecularly imprinted and non-molecularly imprinted): β–cyclodextrin hydrate (98%), cholesterol (95%), and tolylene 2,4-diisocyanate (95%), all from Aldrich. Dimethyl sulfoxide (A.C.S. Reagent) was purchased from J.T. Baker.

Alcohol denatured (Fisher Scientific), acetone (J.T. Baker, A.C.S. Reagent), tetrahydrofuran, minimum 99% (Sigma), milli-Q Academic deionized water, methanol (EM Science, HPLC Grade), and toluene (EM Science, A.C.S. Reagent) were the solvents used.

The silylating reagents used were trimethylsilyl chloride and hexamethyldisilazane (both from Supelco). Dichlorodimethylsilane (Sigma, >99.5%) was used to deactivate the glassware. 5α–Cholestane (Sigma) was used as the internal standard when performing gas chromatography experiments.

The various fluorophores used were, naphthalene, (puriss Fluka, >99%), 2-naphthol (Aldrich, 99%), nabumetone (Sigma), and DL-propranolol hydrochloride (Sigma).

All chemicals were used as received.

2.2 Instrumentation

Gas chromatography analyses were performed on a Perkin Elmer Auto System XL Gas Chromatograph, equipped with a flame ionization detector. The oven and injector were set at 285°C and 340°C, respectively. Helium and compressed air were used as carrier gases and the flow rate was 40.0mL/minute. The column used was the Supelco Equity 1, 30m/0.25mm/250μm. The injection volume remained consistent at 2μL.

Fluorescence intensity was analyzed and measured using a Perkin Elmer LS 50B Luminescence Spectrometer. Measurements were made in 1 cm by 1 cm standard quartz cuvettes (Hellma).

When performing re-binding experiments, solutions were agitated at a setting of four using a VWR S-500 Orbital Shaker.

Several items (i.e., β–cyclodextrin, cholesterol, and all glassware) were dried at 70°C at a reduced pressure in a vacuum oven (Fisher Scientific).

2.3 Polymer Synthesis Procedures

2.3.1 Cholesterol-Templated Molecularly Imprinted Polymer

The method used here was similar to the molecular imprinting technique by Komiyama *et. al.*[23,34-37,44,45] Initially, 5.00g (0.0044mol) of β–cyclodextrin (β–CD) and 0.58g (0.0015mol) of cholesterol were dried overnight *in vacuo* at 70°C. A bulk quantity of dimethyl sulfoxide (DMSO) was dried overnight using 4Å molecular sieves. All glassware used during synthesis was thoroughly cleaned with soap and water, and rinsed with distilled water and acetone, and allowed to dry overnight in the vacuum oven. 50mL of dried DMSO was measured using a 100mL graduated cylinder and transferred to a 100mL beaker containing a magnetic stir bar and placed on a hotplate set at 70°C. The β–CD was added slowly in order to avoid the formation of aggregates. Once all of the β–CD was dissolved, the temperature of the hotplate was decreased to 65°C, at which time cholesterol was added to the solution. Using a pipette, 7mL (0.0486mol) of tolylene 2,4-diisocyanate (TDI) was measured and gradually added to the solution. The solution was magnetically stirred over heat and a solid gel formed after 30 minutes. The beaker containing the cholesterol molecularly imprinted polymer was allowed to cool to room temperature before any further processing. Once cooled, the polymer was chopped into small pieces within the beaker using a scoopula. Then the polymer was washed with 1L of acetone via vacuum filtration. Acetone was used in order to remove any unreacted TDI and DMSO. The chopped polymer was then ground to a granular consistency using a mortar and pestle. The polymer was subsequently washed with 500mL of hot milli-Q distilled water to remove any unreacted β–CD. In order to remove the cholesterol, the polymer was magnetically stirred in 100mL of tetrahydrofuran (THF) for one hour and then vacuum filtered. This wash step was done in triplicate, which was sufficient in order to remove all of the cholesterol that was added initially to form the polymer. To confirm the removal of cholesterol a sample from the filtrate was taken after each wash period in order to quantify the cholesterol concentration using gas chromatography

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(GC). Finally, 500mL of ethanol was used to wash the polymer via vacuum filtration, at which time the molecularly imprinted polymer was dried *in vacuo* at 65°C for 24 hours before being used.

2.3.2 Non-Molecularly Imprinted Polymer

Synthesis of non-molecularly imprinted polymers was very similar to molecularly imprinted polymers. The only difference is no cholesterol was added when making non-molecularly imprinted polymers.[23,34-37,44,45] In addition, there was a difference in the length of time it took the NMIP gel to form, which was 45 minutes.

2.4 Calibration Curve Methods

2.4.1 Cholesterol Calibration Curve in THF

Volumetric flasks and pipettes were all thoroughly cleaned with soap and water, and rinsed with distilled water, acetone and THF, and dried before use. A stock solution of cholesterol was made by weighing and transferring 0.1160g of cholesterol into a 100mL volumetric flask and diluting to the mark with THF. The concentration of the stock solution was 0.0030M. Another stock solution was made for the internal standard by weighing and transferring 0.0110g of 5α–cholestane to another 100mL volumetric flask and adding enough THF to afford a final concentration of 0.0003M. Eleven standard dilutions of the standard were made in 10mL volumetric flasks using appropriate pipettes. The concentrations of those standard dilutions were: 0, 0.0003, 0.0006, 0.0009, 0.0012, 0.0015, 0.0018, 0.0021, 0.0024, 0.0027, and 0.0030M. Using a 2mL pipette, 0.5mL of each standard dilution was measured and added to 2mL GC vials. Using another 2mL pipette, 0.5mL of the solution of the internal standard was measured and added to each of the GC vials. In order to reduce tailing in the chromatograms from the GC and to reduce the run time, each solution was derivatized as outlined in Section 2.5 (see below). Subsequently, each solution was run on the GC under the conditions described in Section 2.2.

2.4.2 Cholesterol Calibration Curve in THF and Water Solution

Volumetric flasks and pipettes were all thoroughly cleaned with soap and water, and rinsed with distilled water, acetone and THF, and dried before use. A stock solution of cholesterol was made by weighing and transferring 0.1160g of cholesterol into a 100mL volumetric flask and adding 55mL of THF using a pipette and then filling to the mark with milli-Q distilled water. The final stock solution had a concentration of 0.0030M in a 55:45 (vol/vol) THF/water solution. A stock solution of the internal

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standard was also made by the same method described in 2.4.1 in THF. Eleven standard dilutions were made in 10mL volumetric flasks using appropriate pipettes and filling to the mark with a 55:45 (vol/vol) THF/water solution. The concentrations of those standard dilutions were: 0, 0.0003, 0.0006, 0.0009, 0.0012, 0.0015, 0.0018, 0.0021, 0.0024, 0.0027, and 0.0030M. Using a 2mL pipette, 0.5mL of each standard dilution was measured and transferred to a 2mL GC vial. Using another 2mL pipette, 0.5mL of the internal standard was measured and added to each of the GC vials. Finally, each solution was run on the GC under the conditions described in Section 2.2.

2.5 Derivatization of Cholesterol

In order to reduce tailing seen on the chromatograms and to reduce the long run times of each cholesterol solution, silylating reagents were used to derivatize cholesterol. Each GC vial containing a cholesterol solution in THF only, was treated by adding 0.5mL of hexamethyldisilazane via a 100μL syringe. Using a 10μL syringe, 20μL of trimethylsilyl chloride was measured and added to each GC vial. The vials were capped, shaken and heated at 55°C for 20 minutes. The vials were removed, and allowed to cool to room temperature before GC analysis.[46]

2.6 Deactivation of Glassware

All GC vials used for derivatizing cholesterol samples were deactivated and kept dry prior to being used. A 11% solution of a derivatizing reagent was made by using 10mL of dichlorodimethylsilane and 80mL of toluene. Each GC vial was filled with this solution and allowed to sit for 20 minutes. The contents were emptied and rinsed twice with toluene and then with methanol. The vials were dried and kept in a desiccator until needed.[47]

2.7 Re-Binding Experiments: Gas Chromatography Measurements

2.7.1 Cholesterol in THF

All glassware was thoroughly cleaned with soap and water, and rinsed with distilled water and acetone, and dried before performing any experiments. Screw cap jars were used in this experiment. 0.1160g of cholesterol was weighed out and transferred to each jar. 100mL of THF was measured using a 100mL graduated cylinder and transferred to each jar containing cholesterol. Cholesterol molecularly

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imprinted polymers will be referred to as MIP, and non-molecularly imprinted polymers will be referred to as NMIP. 1.160g, 4.000g or 8.000g of MIP or NMIP were weighed and added to the appropriate jar. Each jar was shaken on an orbital shaker according to the method described in Section 2.2. The solutions were allowed to shake for one hour intervals, after which 0.5mL of supernatant was taken using a 100μL syringe and transferred to a deactivated GC vial. 0.5mL of the internal standard solution was also added to each vial at which time the contents were derivatized using the method described in Section 2.5. 0.5mL of THF was added to each shaker jar to replace the volume of the aliquot removed for testing before the jar was placed back on the shaker for the next hour. The re-binding experiments described here were done for a total of four hours.

2.7.2 Cholesterol in THF and Water Solution

All glassware was thoroughly cleaned with soap and water, and rinsed with distilled water and acetone, and dried before performing any experiments. Screw cap jars were used in this experiment. 0.1160g of cholesterol was weighed out and transferred to each jar. 55.0mL of THF and 45.0mL of milli-Q distilled water was measured using a 100mL graduated cylinder and transferred to each jar containing cholesterol[34-36]. 1.160g, 4.000g or 8.000g of MIP or NMIP were weighed and added to the appropriate jar. Each jar was shaken on an orbital shaker according to the method described in Section 2.2. The solutions were allowed to shake for one hour intervals, after which 0.5mL of supernatant was taken using a 100μL syringe and transferred to 2mL GC vials. 0.5mL of the internal standard solution was also added to each vial. In alternation, 0.5mL of THF or milli-Q distilled water was added to each jar before resuming re-binding for the next hour interval in order to replace the volume of the testing aliquot. The re-binding experiments described here were done for a total of four hours.

2.8 Binding Experiments: Fluorescence Measurements

2.8.1 Fluorophore in THF and Water Solution

Each jar contained a 55:45 (vol/vol) solution of THF and milli-Q distilled water, which was measured using a 100mL graduated cylinder. 0.0019g of naphthalene, 0.0020g of 2-naphthol, 0.0034g of nabumetone, and 0.0039g of DL-propranolol hydrochloride were weighed and transferred to individual jars. All fluorophores were soluble in the said solvent systems. The initial fluorescence intensity of the solutions were measured and recorded (see Table 2.1). 1.160g of MIP or NMIP were weighed and added to the appropriate jars, and the solutions were shaken using an orbital shaker following the method described in Section 2.2. After one hour, a sample of the supernatant was taken and the fluorescence

intensity was measured. Before binding resumed, the aliquot was returned to the appropriate solution. Fluorescence intensity was measured every hour for a total of five hours and in some instances for 24 hours.

2.8.2 Fluorophore and Cholesterol in THF and Water Solution for Competitive Re-binding

Each jar contained a 55:45 (vol/vol) solution of THF and milli-Q deionized water, which was measured using a 100mL graduated cylinder. 0.0019g of naphthalene and 0.0020g of 2-naphthol were weighed and transferred to individual jars. All fluorophores were soluble in the said solvent system. Subsequently, 0.0580g of cholesterol was weighed and added to each jar. The initial fluorescence intensity of the solutions was measured and recorded (see Table 2.1). 1.160g of MIP or NMIP were weighed and added to the appropriate jars and the solutions were shaken using an orbital shaker following the method described in Section 2.2. After one hour, a sample of the supernatant was taken and the fluorescence intensity was measured. Before re-binding resumed, the aliquot was returned to the appropriate solution. Fluorescence intensity was measured every hour for a total of five hours and then at 24 hours.

2.8.3 Fluorophore Displacement with Cholesterol in THF and Water Solution

Each jar contained a 55:45 (vol/vol) solution of THF and milli-Q deionized water, which was measured using a 100mL graduated cylinder. 0.0019g of naphthalene was weighed and transferred to individual jars. The initial fluorescence intensity of the solution was measured and recorded (see Table 2.1). 1.160g of MIP or NMIP were weighed and added to the appropriate jars and the solutions were shaken using an orbital shaker following the method described in Section 2.2. After one hour, a sample of the supernatant was taken and the fluorescence intensity was measured. Before binding resumed, the sample was returned to the appropriate solution. Fluorescence intensity was measured every hour for a total of five hours and then at 24 hours. At the 24 hour time interval 0.0580g of cholesterol was weighed and transferred to both jars and these samples were shaken using the orbital shaker. After one hour, a sample of the supernatant was taken and the fluorescence intensity was measured. Before binding resumed, the aliquot was returned to the appropriate solution. Fluorescence intensity was measured every hour for a total of five hours and then at 24 hours.

Table 2.1 Parameters and Properties for the Four Fluorescent Molecules Used.[48]

Fluorophore	Excitation (nm)	Emission (nm)	Slit Widths	Molecular Weight (g/mol)
Naphthalene	280	337	15 and 15, with 1% attenuation	128.17
2-Naphthol	310	355	7.5 and 7.5, with 1% attenuation	144.19
Nabumetone	320	556	7.5 and 7.5, with 1% attenuation	228.29
DL-Propranolol Hydrochloride	310	337	6.5 and 6.5, with 1% attenuation	259.34

CHAPTER 3

RESULTS

3.1 Polymer Synthesis

3.1.1 Polymer Characterization

The preparation and characterization of the cholesterol-templated MIP and NMIP have been extensively detailed in a series of publications from Komiyama's lab.[35,36,44] Therefore, in the present study we did not do a full characterization of the polymerization products. During the synthesis of the cholesterol imprinted and non-imprinted polymers a number of observations were made that are characteristic of polymerization. When synthesizing the MIP, the initial polymerization solution took on a thick, gel-like consistency 30 minutes after the addition of the crosslinker. At this point the magnetic stir bar could no longer spin. When synthesizing the NMIP, it took 45 minutes after the addition of the crosslinker for the polymer's consistency to become thick and gel-like. Once both of the solid products were processed and sufficiently dried, they were placed in various solvents including DMSO, THF, and THF and water. Neither product dissolved in any of the three solvents. These observations are consistent with polymerization and we conclude that we have successfully prepared both the cholesterol-templated MIP and the NMIP.

3.1.2 Cholesterol Molecularly Imprinted Polymer Washing

In order to ensure that all the template was removed from the polymer, it was washed with THF, which is a good solvent for cholesterol. Each round of washing was tested by GC to quantify how much cholesterol was in each washing, to ensure the total amount of cholesterol that was washed out corresponded to that which was added initially, and to ensure that there was no cholesterol left within the polymer. Figure 3.1 shows a typical set of chromatograms obtained in the washing experiment.

Deciding on a method to determine and quantify the removal of cholesterol was extremely important because it confirmed that indeed the molecularly imprinted sites within the polymer were vacant and no longer binding the template. In order to calculate the cholesterol concentration within each washing, the equation of the calibration curve was used (Appendix A):

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$$y = 2.5494 \times 10^{09} x \,,$$

where y is the GC peak area and x is the cholesterol concentration

Initially, 0.0015 moles of cholesterol was added when synthesizing the polymer. According to the calculations, 0.0015 moles of cholesterol and therefore 100% of the template was in fact washed out after two consecutive hours of washing in THF.

Table 3.1 Cholesterol Concentrations in MIP Washings with THF.

Washing Cycle (per hour)	Area (μVsec)	Cholesterol Concentration (mol/L)
1	2467682	0.00097
2	1406106	0.00055
3	0	0.00000
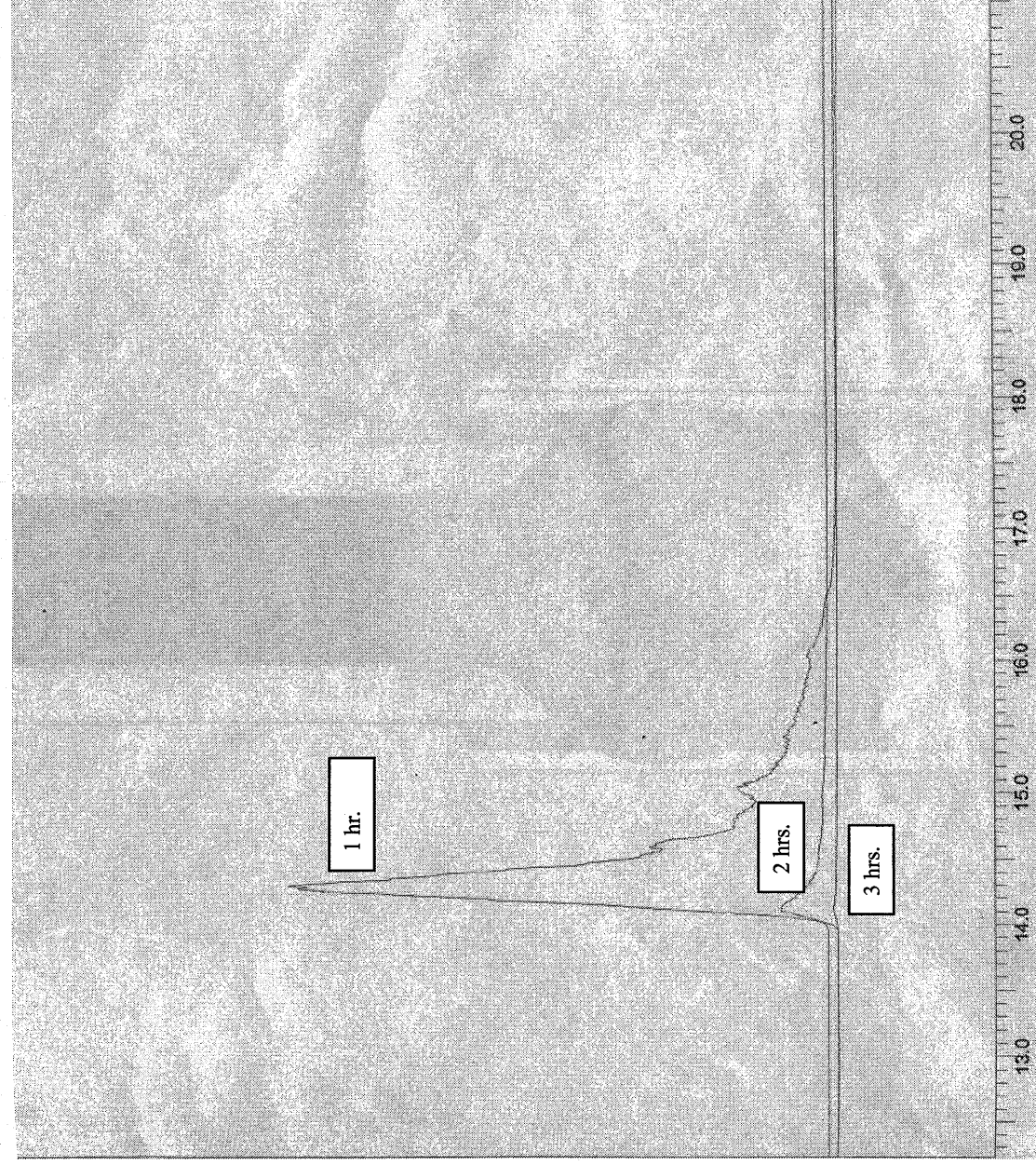
Figure 3.1 Chromatograms of Cholesterol MIP Washing

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3.2 Re-Binding Experiments Using Gas Chromatography

3.2.1 Cholesterol Re-Binding in THF

The re-binding experiments performed were done in order to provide evidence that cholesterol could specifically bind to its tailor-made receptors within the polymer matrix of a fully "de-templated" MIP, compared to the control NMIP. These experiments were done in THF, since cholesterol is highly soluble in this particular solvent. The duration of the re-binding experiment was four hours in total. In addition, three different amounts of MIP were used in order to examine if a greater quantity of polymer, and therefore a greater quantity of binding sites, would have an effect on the degree of cholesterol binding over time.

As illustrated in Figure 3.2, regardless of the quantity of MIP used for re-binding there is no evidence to suggest re-binding of cholesterol by the MIP in THF. The cholesterol concentration in the supernatant does not decrease over time, but rather fluctuates, leading to the scattered appearance of the data points. Furthermore, increasing the amount of polymer does not promote the uptake of cholesterol from the solution and thus does not increase the degree of re-binding over time.

In Figure 3.3, the results of the re-binding experiments using NMIP are displayed. Again the data suggests there is little or no binding of cholesterol to the NMIP.

Figure 3.2 Change in Cholesterol Concentration in Supernatant Over Time During Re-Binding with Various Amounts of MIP in THF.

Figure 3.3 Change in Cholesterol Concentration in Supernatant Over Time During Re-Binding with Various Amounts of NMIP in THF.

3.2.2 Cholesterol Re-Binding in THF and Water Solution

Re-binding experiments were also done in a 55:45 (vol/vol) THF and water solution[34-37,44]. Changing the solvent system from an environment where cholesterol is highly solubl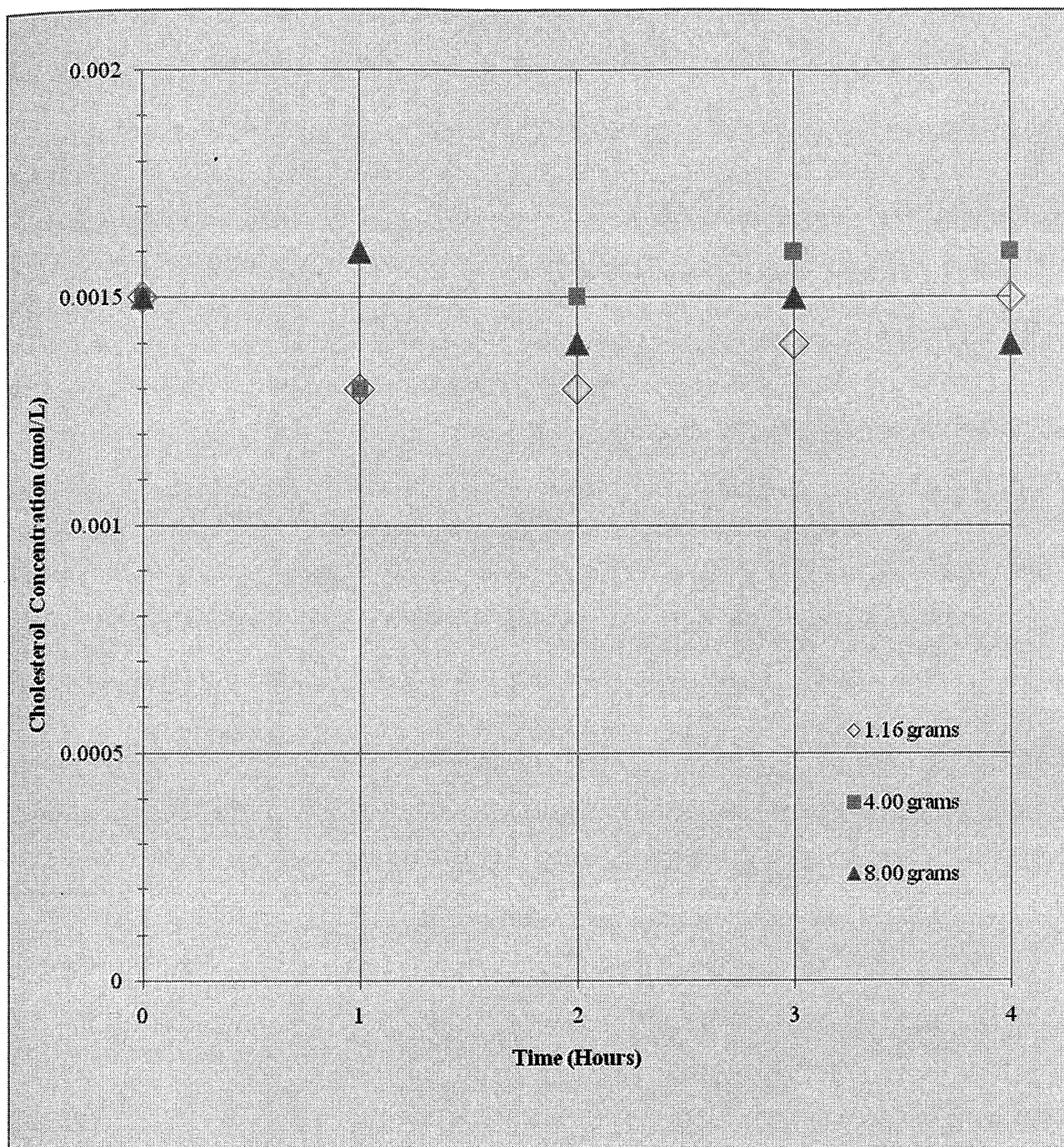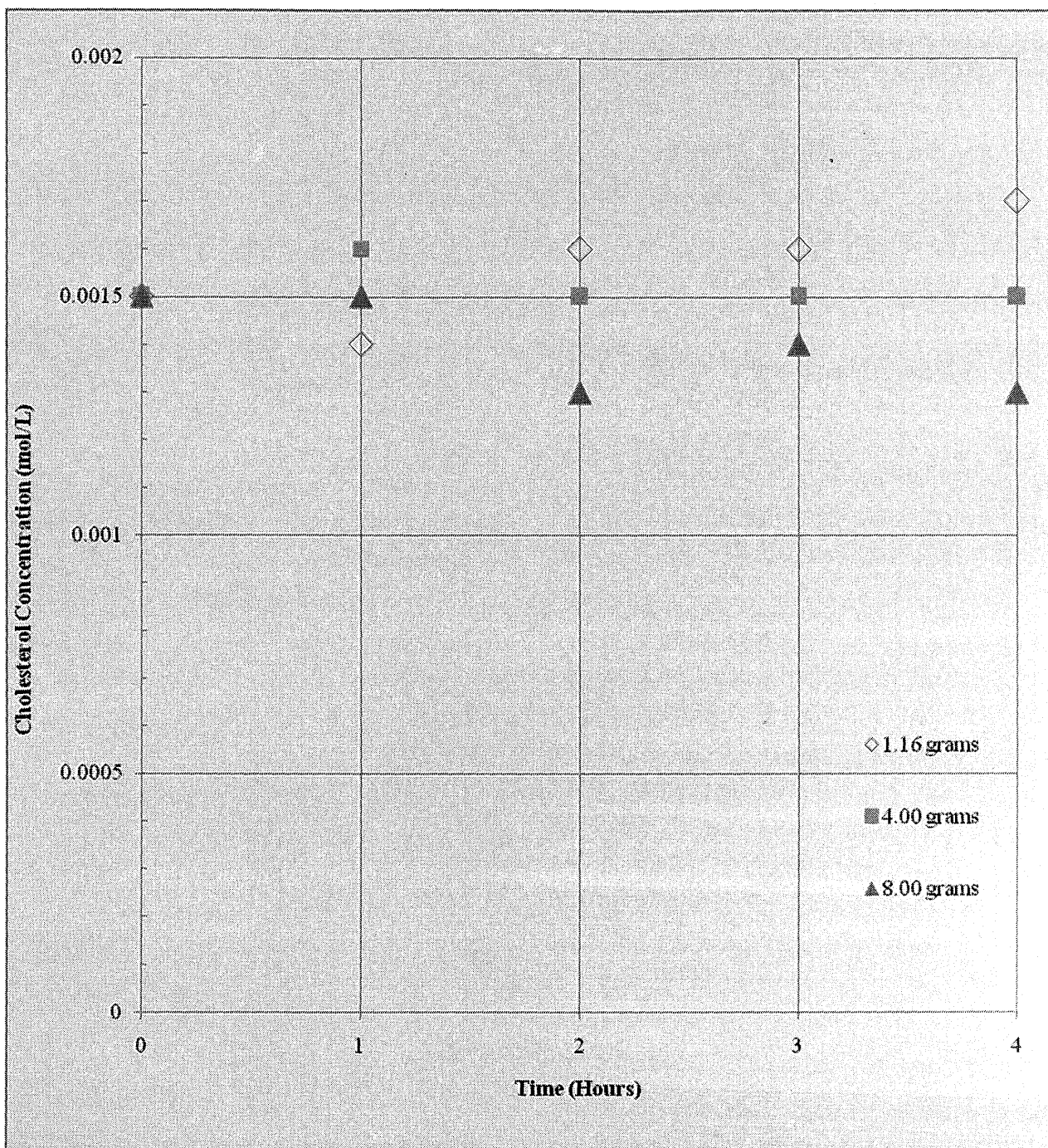e to a more polar environment may enhance its interaction with the β–CD cavities within the polymers and promote re-binding. Cholesterol dissolved within this mixed solvent even though it is known to be very insoluble in water. Similar to the re-binding experiments done in THF, the experiments lasted for a total of four hours, and a sample was taken every hour in order to quantify the cholesterol concentration in the supernatant solution, and to calculate the degree of cholesterol re-bound by the polymers. Various quantities of polymer were again used to clarify if in fact this could enhance the degree of re-binding.

Once again, the results (Figure 3.4) do not show a downward trend with respect to cholesterol concentration over time. The scattered appearance of the cholesterol concentration provides additional support that the MIPs are not specifically, nor strongly binding their template. The NMIP (Figure 3.5) also shows little or no re-binding, which was expected.

Table 3.2 displays the percent of cholesterol re-bound by MIP and NMIP at the various masses used in the re-binding experiments. When 1.16 grams and 8.00 grams of polymer were used to re-bind cholesterol, the MIP bound the same amount of the template as the NMIP. When 4.00 grams of polymer was used, the NMIP bound more cholesterol than the MIP, and in this case zero percent of cholesterol was taken up by the MIP. Thus, cholesterol molecularly imprinted polymers show the same, if not less binding efficiency for its template compared to non-molecularly imprinted polymers, suggesting that the change in re-binding environment did not enhance the degree of cholesterol re-binding. Furthermore, increasing the amount of polymer did not enhance the degree of cholesterol re-binding.
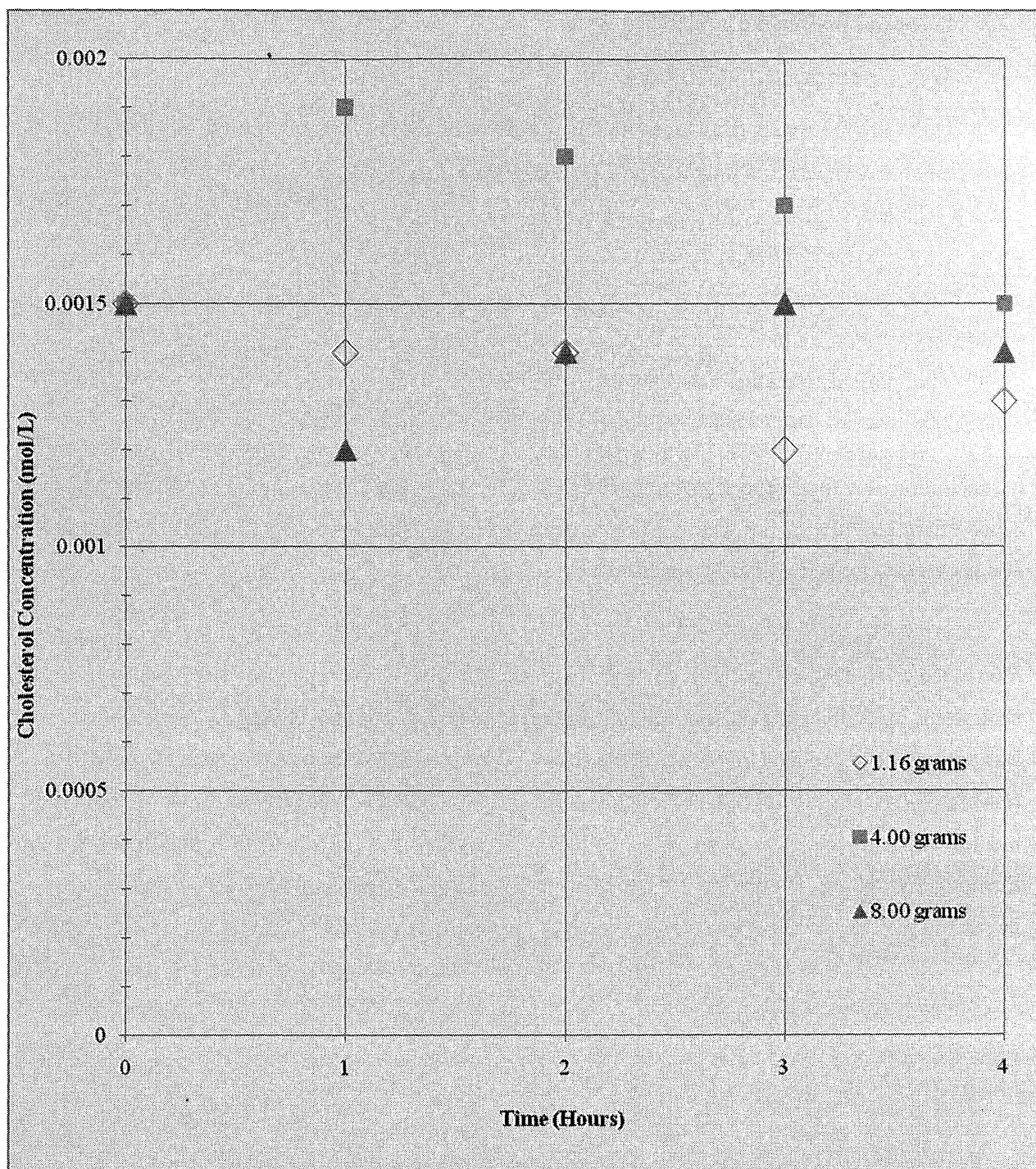
Figure 3.4 Change in Cholesterol Concentration in Supernatant Over Time During Re-Binding with Various Amounts of MIP in THF and Water.
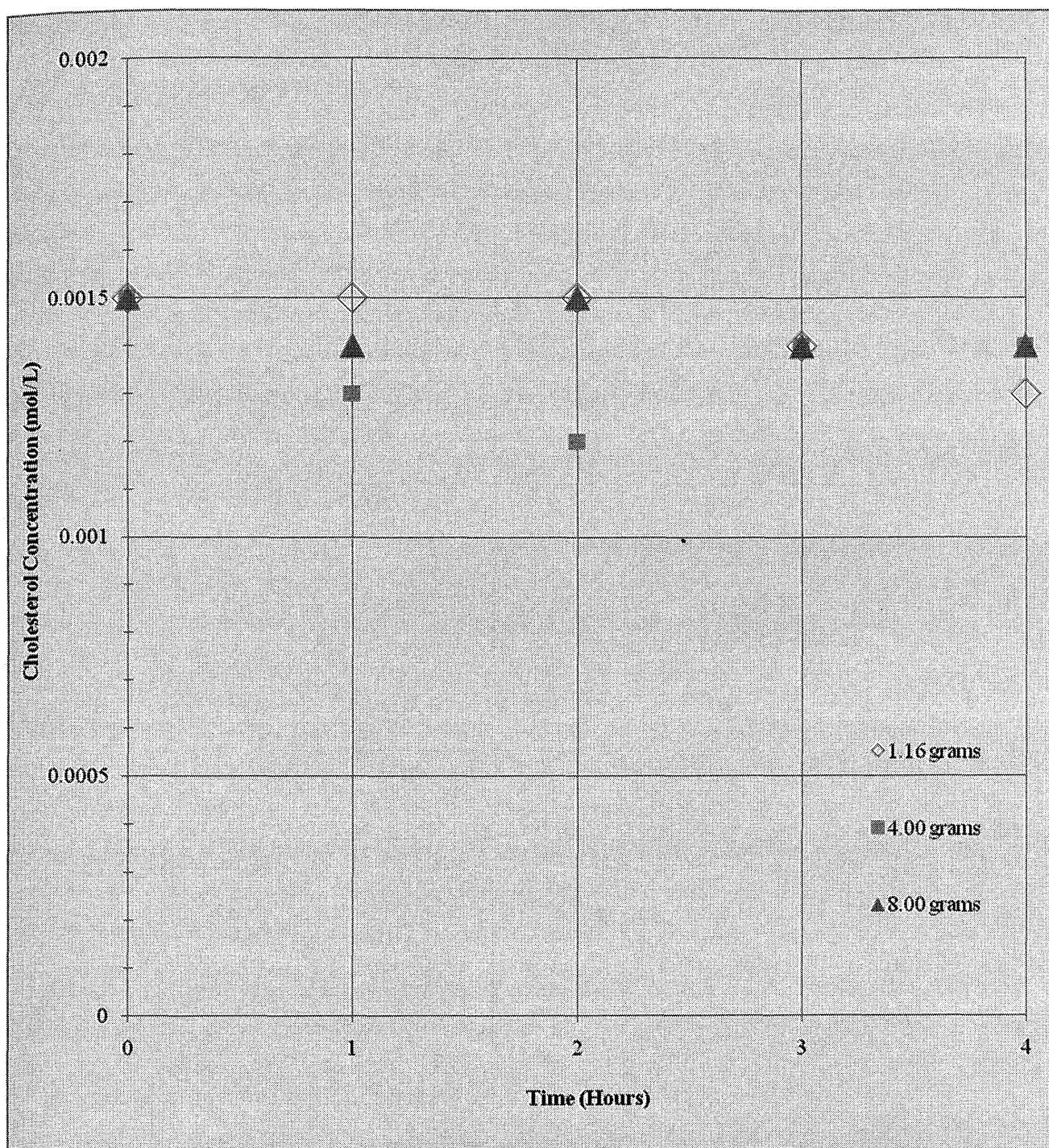
Figure 3.5 Change in Cholesterol Concentration in Supernatant Over Time During Re-Binding with Various Amounts of NMIP in THF and Water.

Table 3.2 Total Percentage of Cholesterol Re-Bound by MIP and NMIP in THF and Water After 4 Hours of Equilibriation.

Percent of Cholesterol Re-Bound		
Amount of Polymer Used in Re-binding (grams)	MIP	NMIP
1.16	13.3%	13.3%
4.00	0%	6.7%
8.00	6.7%	6.7%

3.3 Binding Experiments Using the Luminescence Spectrometer

3.3.1 Fluorophore in THF and Water Solution

A number of fluorophores (Figure 3.6) known to complex with β–CD were used as alternatives for cholesterol in binding experiments, as described earlier. Opting for fluorescent guests structurally unrelated to cholesterol was done partly to test whether the polymers' binding sites were available and perhaps not blocked in any way, and to test the specificity of the cholesterol-templated MIP. Another reason for using the fluorophores is that fluorescence is a much more sensitive analytical tool than the GC. The four fluorophores used were naphthalene, 2-naphthol, nabumetone, and propranolol HCl. Note that the same mass of polymer (1.16 grams) was used in all these experiments.

When naphthalene was used as the guest, there was a significant decrease in its fluorescence intensity in the supernatant over time, suggesting it was complexing with the polymers. Referring to Figure 3.7, most of the guest was bound by the fourth hour, after which there was minimal change in intensity. This implies that the polymers were saturated with the fluorophore by the fourth hour. What is interesting is that the NMIP binds the naphthalene more than the MIP does. According to Table 3.3, NMIP binds naphthalene 17.7% more than the MIP.

The results in Figure 3.8, also illustrate a downward trend in 2-naphthol's fluorescence intensity over time. By hour four the MIP and NMIP have reached saturation and the intensity remains unchanged beyond this point. Similar to the findings with naphthalene, the NMIP binds more of the fluorophore than the MIP. Table 3.3, shows that 8.1% of 2-naphthol was bound by the cholesterol-templated MIP, where approximately 20% was bound by the NMIP. Therefore, the NMIP bound twice as much of the fluorescent molecule compared to the MIP.

Nabumetone, which is larger than naphthalene and 2-naphthol, showed essentially no binding to the cholesterol MIP and NMIP (Figure 3.9).

Propranolol HCl showed a similar degree of polymer binding as 2-naphthol (Figure 3.10). With respect to the MIP, there was a slight change in intensity over time with a total of 5.0% bound. On the other hand, NMIP was four times more efficient in binding propranolol HCl than the MIP. NMIP was able to bind approximately 20% of the fluorophore in the supernatant after four hours.

Comparing the values of fluorophore percent binding (Table 3.3), naphthalene exhibits the greatest binding interaction towards cholesterol MIPs and NMIPs. Naphthalene is also one of the smallest and

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least polar of the four fluorophores studied. Nabumetone shows the lowest percent binding values, and is one of the largest molecules used and is one of the most polar. Once again, NMIPs display a greater affinity to bind to the fluorophores when compared to the cholesterol-templated MIPs, except when nabumetone was used. Nabumetone was the only fluorophore in which the MIP bound more of the guest than the NMIP, but the difference is marginal at approximately 1%.
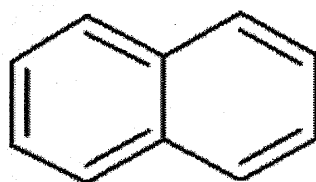
Naphthalene
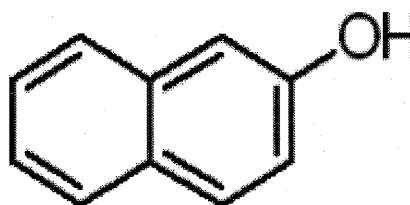
2-Naphthol
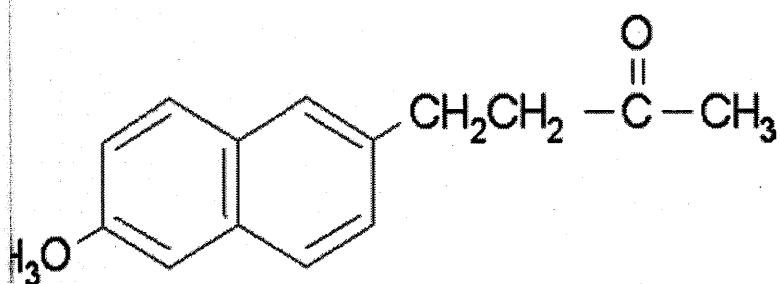
Nabumetone
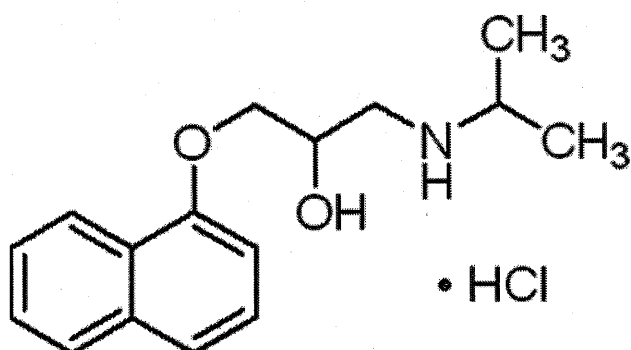
Propranolol HCl

Figure 3.6 Structures of the Four Fluorophores Used.[55]
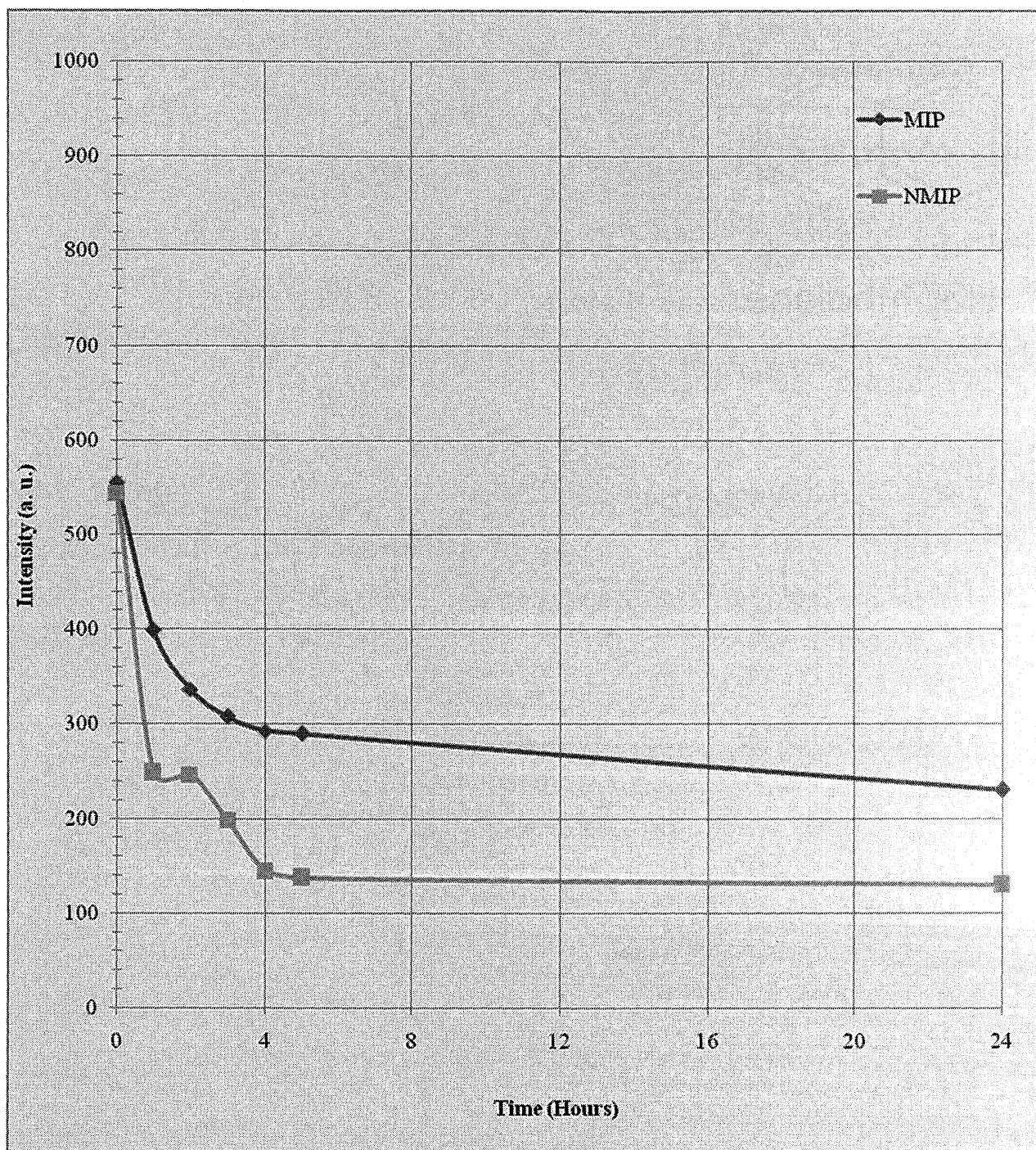
Figure 3.7 Binding of Naphthalene to Cholesterol-Templated MIP and NMIP in THF and Water Solution.
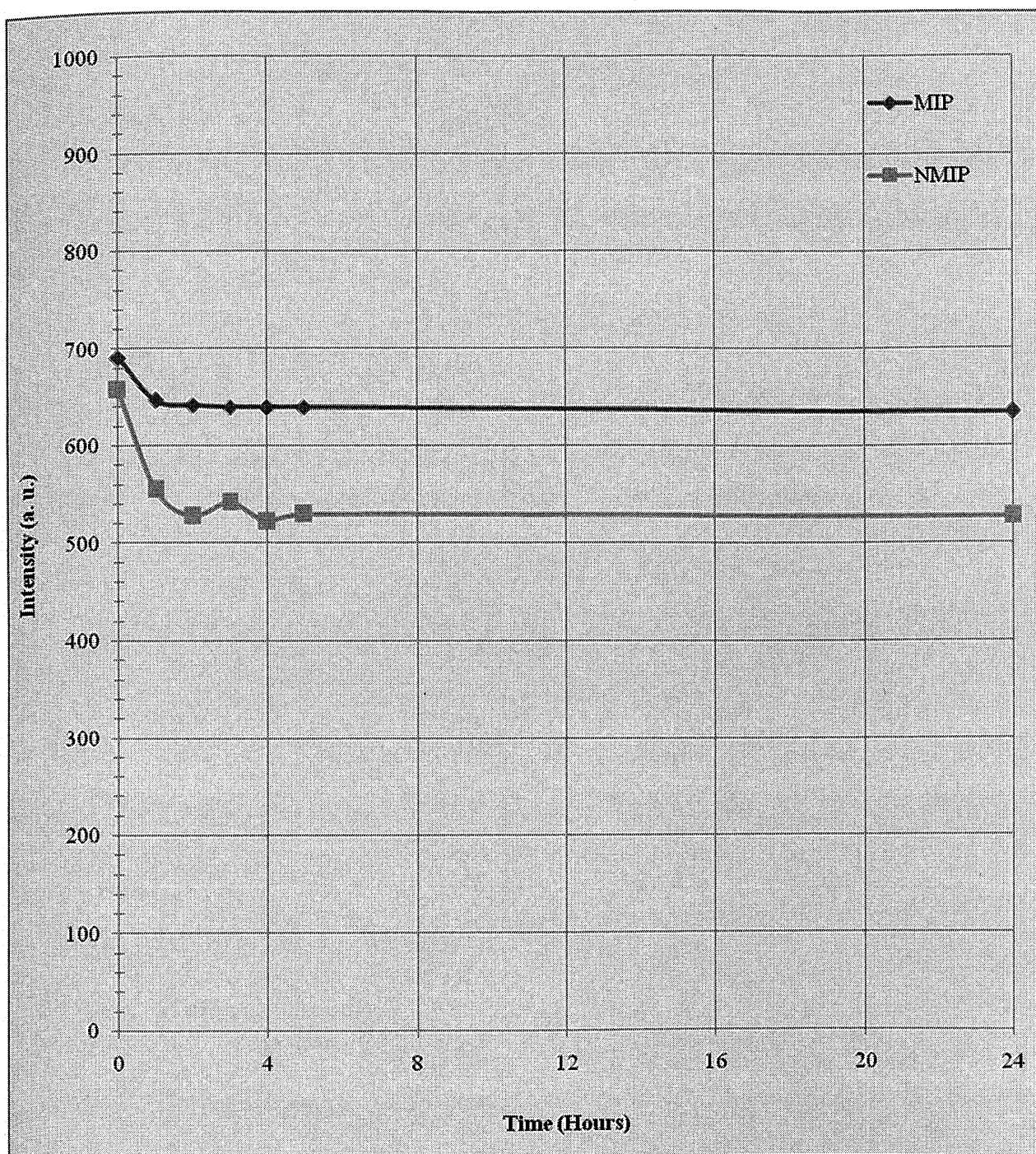
Figure 3.8 Binding of 2-Naphthol to Cholesterol-Templated MIP and NMIP in THF and Water.
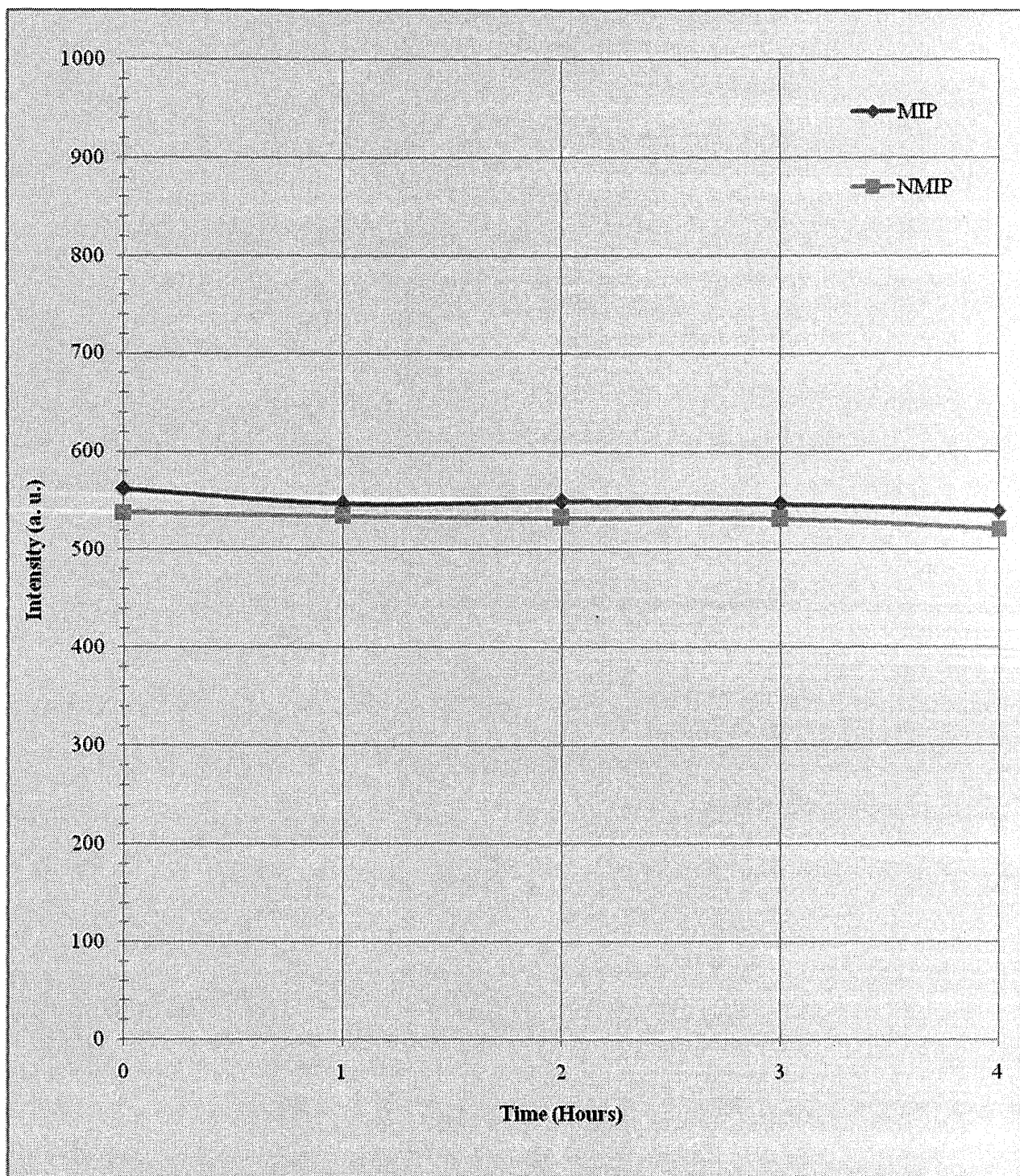
Figure 3.9 Binding of Nabumetone to Cholesterol-Templated MIP and NMIP in THF and Water.
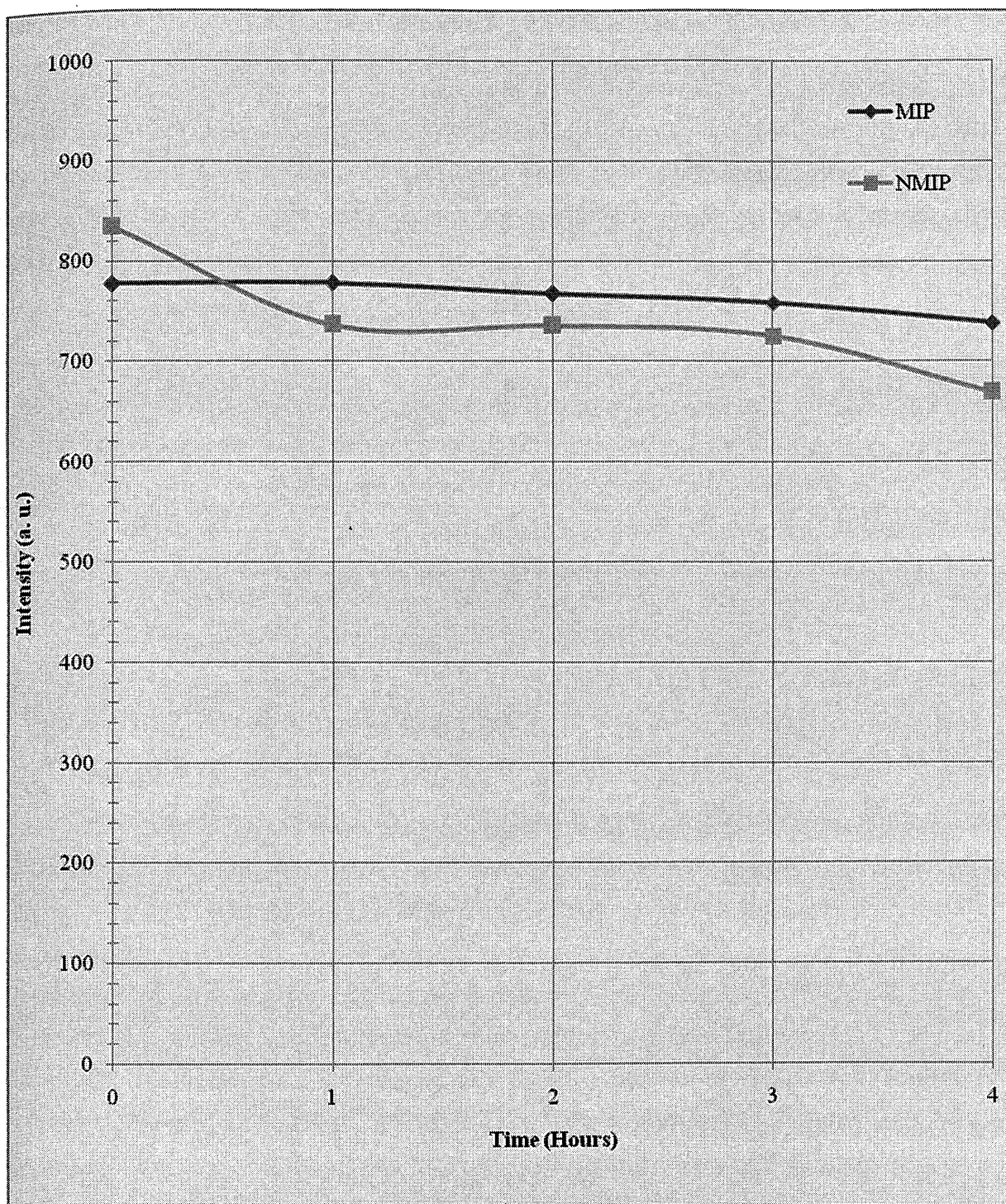
Figure 3.10 Binding of Propranolol HCl to Cholesterol-Templated MIP and NMIP in THF and Water.

Table 3.3 Total Percentage of Fluorophore Bound by Cholesterol-Templated MIP and NMIP in THF and Water Solution Over the Entire Duration of the Experiment.

Fluorophore	Percent of Fluorophore Bound	
	Cholesterol-templated MIP	Cholesterol-templated NMIP
Naphthalene	58.4%	76.1%
2-Naphthol	8.1%	19.8%
Nabumetone	4.1%	3.3%
Propranolol HCl	5.0%	19.8%

3.3.2 Competitive Re-Binding Between Fluorophore and Cholesterol in THF and Water Solution

As an alternative method to check whether or not cholesterol could re-bind to its MIP, competitive re-binding experiments with cholesterol and fluorescent molecules were carried out. These tests were an indirect way to measure if cholesterol would bind to its tailor-made host or, if the fluorophore would block it from doing so. Naphthalene and 2-naphthol were chosen as the competitors since they showed the greatest degree of binding with the cholesterol MIPs and NMIPs.

Figure 3.11 is similar in its appearance to Figure 3.7, in that there is a dramatic decrease in the naphthalene fluorescence intensity in the supernatant over time. This suggests that naphthalene continues to bind to the two different sets of polymers, even in the presence of cholesterol. The MIP is able to bind 46.8 % of the fluorophore within 24 hours and the NMIP is able to bind 55.0% of the fluorophore within that same time. Also, the polymers are saturated by the fourth hour of the experiment. Likewise, the NMIP binds naphthalene more so than the MIP which is demonstrated by the greater decrease in intensity over time.

When the competitive re-binding experiments were done with 2-naphthol, a similar outcome was observed (Figure 3.12). There was a decrease in 2-naphthol's intensity in the supernatant in the presence of MIP and NMIP, and both polymers were saturated by the fourth hour. Once again, NMIP bound a greater degree of fluorophore than MIP, with NMIP binding 17.2% and MIP only binding 6.2%.

However, taking a closer look at the numbers displayed in Tables 3.4 and 3.5, the presence of cholesterol may have had a small impact on the degree of fluorophore binding to the cholesterol-template MIP and NMIP. It seems that when cholesterol is competitively re-binding with either fluorophore there is a slight decrease in the percent of fluorophore binding to MIP and NMIP compared to the situation where cholesterol is absent. The implication is that there may be a minimal degree of binding by cholesterol to its MIP and to the NMIP, therefore blocking the naphthalene and 2-naphthol from complexing with the polymers. For example, when binding experiments were done with naphthalene alone, the MIP bound approximately 58% of the guest, but in competition with cholesterol only 47% of the fluorophore was bound. The greatest difference is seen during binding experiments involving naphthalene and NMIP. Approximately 21% more unbound fluorophore is seen when cholesterol is present. This can be interpreted by the cholesterol binding to the polymer, therefore interfering with naphthalene's ability to interact with all of the sites within that polymer. With respect to 2-naphthol, the overall binding was modest in all cases and the differences in the intensity with cholesterol and without cholesterol were small at just 1.9% and 2.6% for MIP and NMIP, respectively.
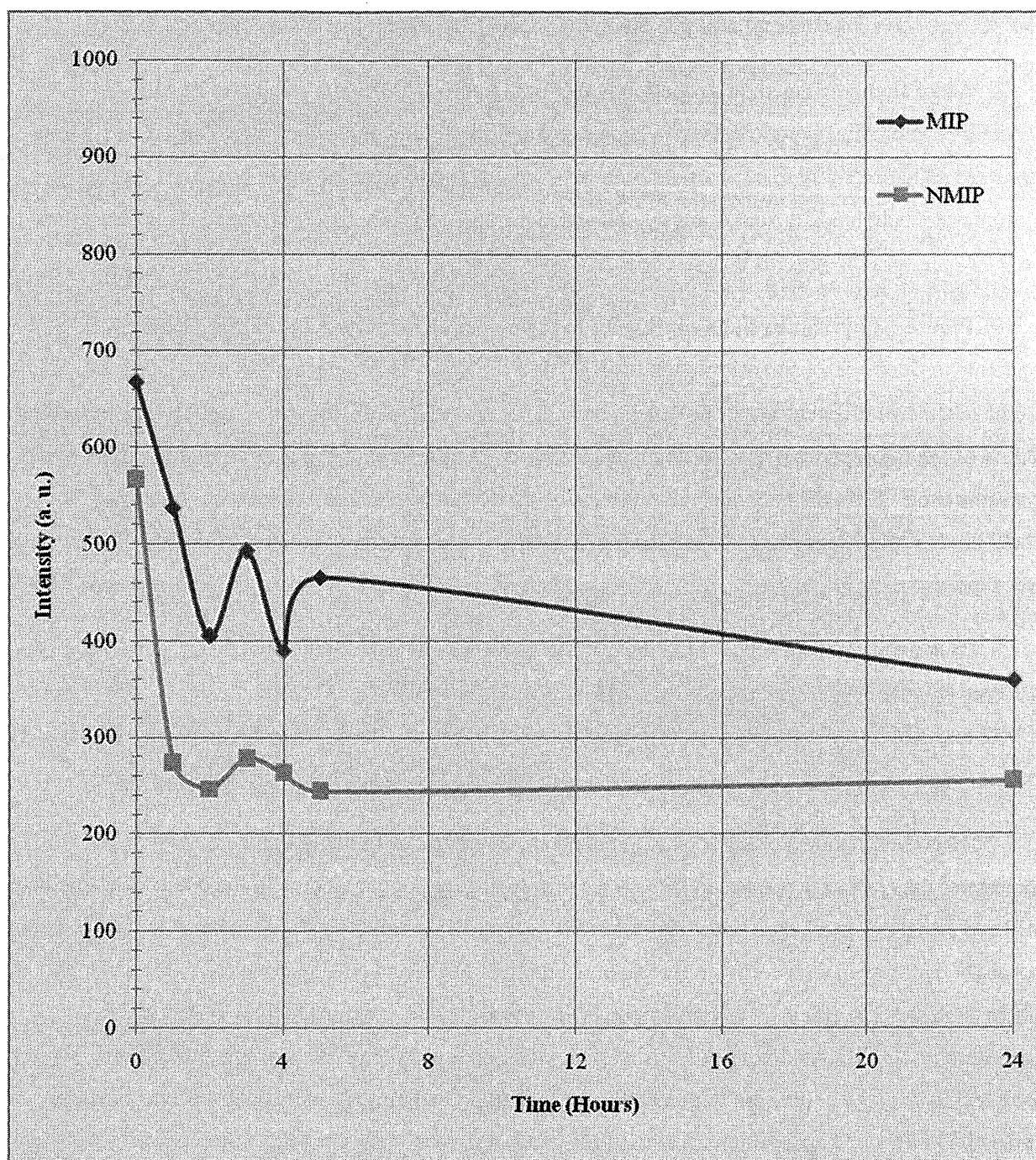
Figure 3.11 Competitive Re-Binding of Cholesterol and Naphthalene by Cholesterol-Templated MIP and NMIP in THF and Water Solution.
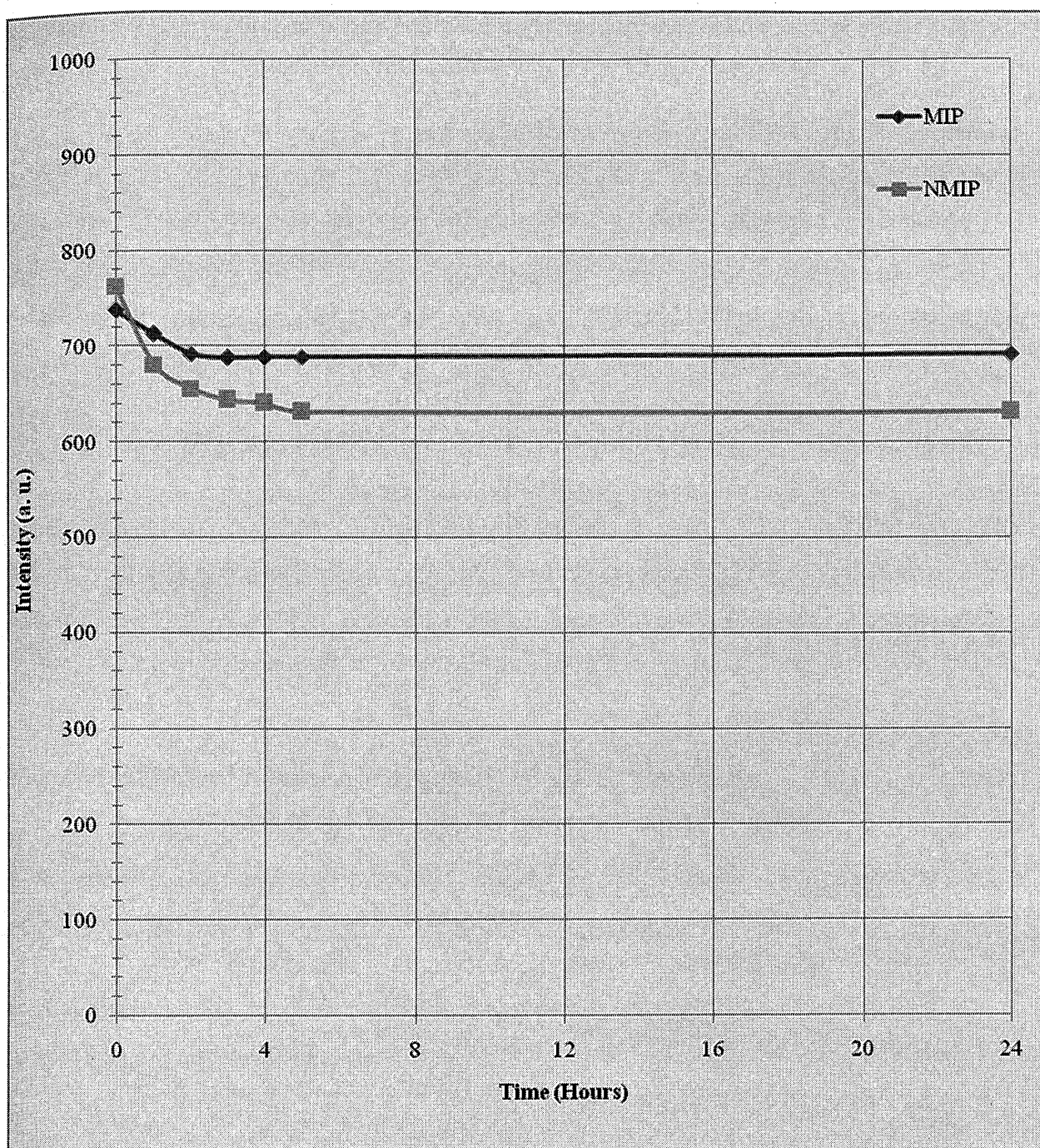
Figure 3.12 Competitive Re-Binding of Cholesterol and 2-Naphthol by Cholesterol-Templated MIP and NMIP in THF and Water Solution.

Table 3.4 Total Percentage of Naphthalene Bound By Cholesterol-Templated MIP and NMIP Alone or in Competition with Cholesterol in THF and Water Solution.

	Competitive Re-binding With Cholesterol	Binding Without Cholesterol	Difference
MIP	46.8%	58.4%	11.6%
NMIP	55.0%	76.1%	21.1%

Table 3.5 Total Percentage of 2-Naphthol Bound By Cholesterol-Templated MIP and NMIP Alone or in Competition with Cholesterol in THF and Water Solution.

	Competitive Re-binding With Cholesterol	Binding Without Cholesterol	Difference
MIP	6.2%	8.1%	1.9%
NMIP	17.2%	19.8%	2.6%

3.3.2 Fluorophore Displacement with Cholesterol in THF and Water Solution

Naphthalene was used again in a binding study with cholesterol-templated MIP and NMIP for a total of 24 hours. We were curious to see whether the addition of cholesterol after naphthalene bound to the polymers would have some effect. In other words, would the presence of the template displace the fluorophore from the sites within the polymers? This would be seen by a subsequent increase in the fluorescence intensity in the supernatant between hours 24 and 48.

The results in Figure 3.13 show the contrary, in that the addition of cholesterol at the 24th hour had no effect on naphthalene's fluorescence intensity during the following 24 hours. The cholesterol-templated MIP bound approximately 44% of naphthalene in the supernatant by hour 24, however approximately 6% of the fluorophore remaining in the supernatant was bound to the MIP by hour 48. Therefore, the cholesterol-templated MIP continued to bind to naphthalene in spite of the addition of its template. NMIP bound approximately 67% of the fluorophore within the first 24 hours, which was 23% more than the MIP. After the addition of cholesterol only 2% of naphthalene remaining in the environment bound to the NMIP. The results collected from this experiment coincide with the previous results that suggest the cholesterol-templated MIP lack specific and selective tendencies for its template.
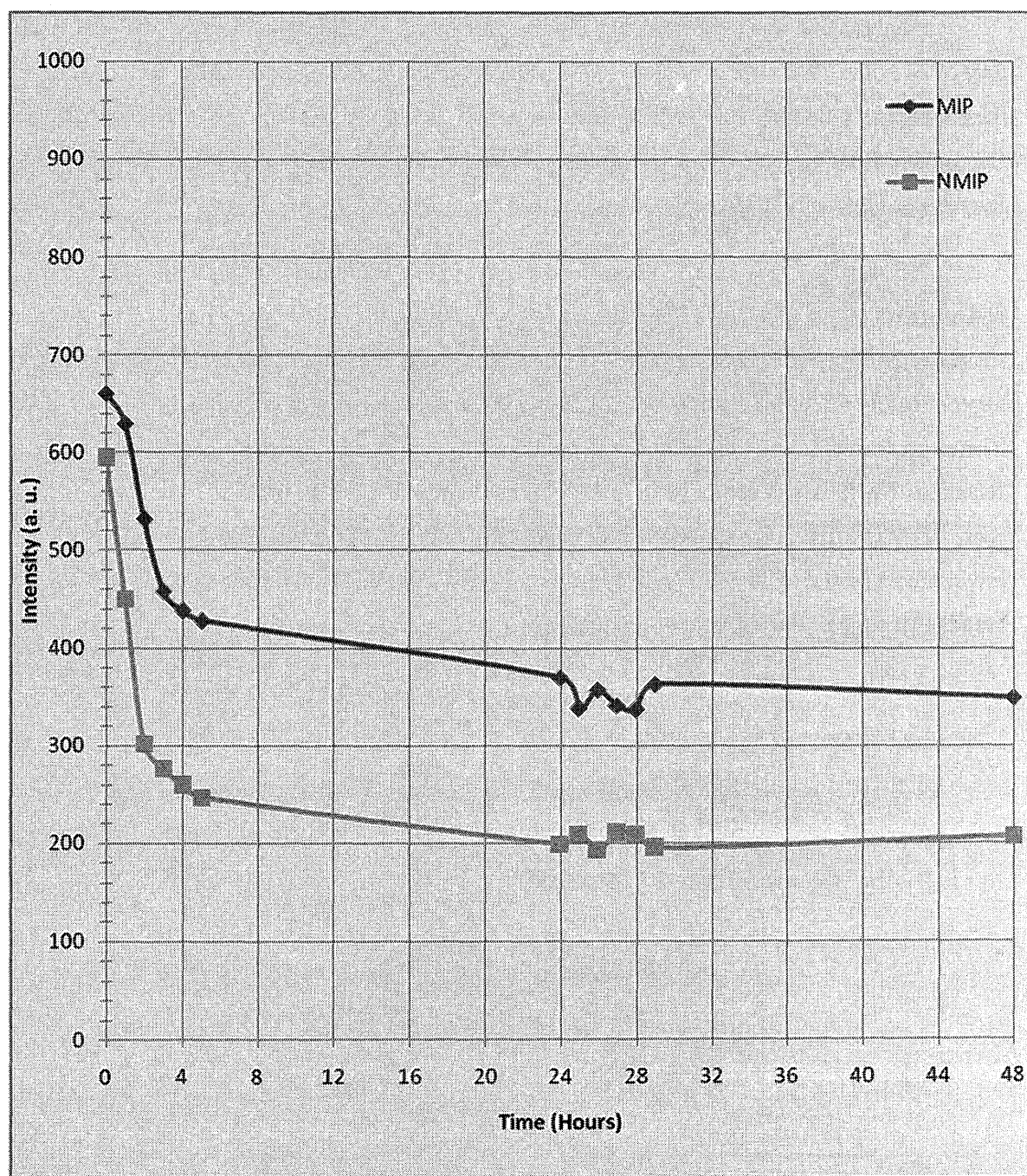
Figure 3.13 Naphthalene Binding with Cholesterol-Templated MIP and NMIP (Hours 0-24) and the Addition of Cholesterol (Hours 24-48) in THF and Water Solution.

CHAPTER 4

DISCUSSION

According to the results presented in this thesis cholesterol-templated β-CD based molecularly imprinted polymers are not as specific, nor selective in binding to their template as initially thought. The cholesterol re-binding studies performed in THF alone and in a solution of THF and water both demonstrated little evidence to suggest template binding was occurring. Furthermore, when binding studies were done using fluorescent molecules structurally unrelated to cholesterol, these guests were able to bind to the polymers to various degrees depending on their size, binding affinity to β-CD, and their polarity.

Based on the literature, cholesterol dissolved in the re-binding solution should bind to the pre-organized β–CD sites within the cholesterol-templated MIP. Specifically, the hydroxyl group at carbon 3 and the alkyl chain at carbon 17 play an important role in the molecular recognition of cholesterol by its MIP binding sites.[8] In contrast, when performing the same experiment using the NMIP there should be a lesser degree of cholesterol binding. In theory, NMIPs lack the template imprinted binding sites and are thought to be composed of randomly distributed functional monomers (i.e., β–CD cavities) that are crosslinked, therefore lacking custom made binding sites specific to the size, shape, and binding complementarity of the template.[15] In addition, the molecular imprinting hypothesis implies that increasing the amount of polymer used for re-binding should increase the amount of template binding to the MIP, because there should be more binding sites available for the template, in this case cholesterol, to bind to. With respect to increasing the amount of NMIP used for re-binding experiments, this should have a minimal effect on the amount of cholesterol binding to the polymer.

The MIPs failure to specifically bind its template is demonstrated in Figures 3.2 and 3.3, where the results of cholesterol re-binding experiments performed in THF are presented. The data displayed in Figure 3.2 are scattered, and there is no downward trend in cholesterol concentration in the supernatant over time. Furthermore, increasing the amount of MIP used in re-binding experiments did not increase cholesterol binding as originally expected. For example, when comparing the change in cholesterol

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concentration in the supernatant over the four hours, 8.00 grams of MIP did not bind more cholesterol than when 1.16 grams of MIP were used. The same was true when comparing the change in cholesterol concentration in the supernatant when re-binding studies were done using 1.16 grams and 4.00 grams of MIP.

The results of cholesterol binding to NMIP are shown in Figure 3.3. Once again the data points are scattered over the total four hour experiment. Regardless of the amount of polymer used there is no downward trend in the cholesterol concentration in the supernatant which was expected. What is interesting is that there is very little difference between the results in Figures 3.2 and 3.3, suggesting that the MIP and NMIP have similar cholesterol binding capabilities. The most likely explanation for this outcome is related to the solvent used, THF. Cholesterol is very soluble in ethers, in fact one gram of cholesterol dissolves in 2.8 mL of ether.[49] Therefore, the cholesterol will tend to partition to the solvent and have little incentive to move from THF, in which it is so soluble, into its binding sites within the MIP.

The main driving force for cholesterol to bind to the sites is hydrophobic interactions.[37] Changing the re-binding solvent to one which is less suitable for cholesterol solvation should enhance re-binding of cholesterol to its MIP. The results of re-binding from a THF and water solution are displayed in Figures 3.4 and 3.5. Changing the re-binding environment did not have the positive effect on re-binding that was expected. In addition these results contradicted the literature.[23,34-37,44,45] With respect to the re-binding studies using MIP, there is, at most, very limited binding of cholesterol from this mixed solvent. When 1.16 grams of MIP were used only 13.3% of the total amount of cholesterol in the supernatant was bound. None of the cholesterol in the supernatant was bound when 4.00 grams of MIP were used and only 6.7% of the total amount of cholesterol was bound when 8.00 grams of MIP were used (Table 3.2). From the data reported here it is unclear whether or not cholesterol is binding to the MIP. Furthermore, there was no relationship between increasing the amount of polymer used and enhancing the level of template re-binding.

In the control experiments, again performed in a 55:45 (vol/vol) THF and water solution, 13.3% of the total amount of cholesterol that was in the supernatant bound to the NMIP, which is the exact amount that MIP bound when 1.16 grams was used (Table 3.2). Therefore, there is no difference in the amount of cholesterol binding between MIP and NMIP when 1.16 grams of polymer were used. The same was true when 8.00 grams of NMIP were used. Only 6.7% of the total amount of cholesterol in the supernatant was bound by the NMIP which is identical to the percentage of cholesterol bound by the MIP when 8.00 grams were used. When 4.00 grams of NMIP were used 6.7% of the total amount of cholesterol was bound which was more than when 4.00 grams of MIP was used, since the MIP did not

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bind any cholesterol from the supernatant in total in that particular case. Ultimately, the outcome of the re-binding studies using cholesterol in either of the solutions indicates that cholesterol-templated MIPs lack the ability to specifically re-bind their template because there is no difference in the amount of template bound by the MIP and NMIP.

The similar and low extent of binding of cholesterol to MIP and NMIP suggests that the template is interacting with both via weak, possibly non-specific interactions. This finding is in marked contrast to the work of Komiyama *et al.* reported in a series of publications in recent years.[23,34-37,44,45] In Komiyama's papers, the cholesterol-templated MIP far exceeded the NMIP in its ability to re-bind the template. Our results are especially surprising in that we followed Komiyama's protocols in preparation of the polymers and performed re-binding experiments under essentially identical conditions as his group's work. At this point we do not have a good explanation for these differences in observations. One possible explanation for the limited uptake of cholesterol by the MIP in this work is incomplete removal of cholesterol from the MIP after its preparation. However, our GC analysis of the washings from the MIP show that this is not the case as good mass balance of the recovered cholesterol was observed.

An alternative method to assess the specificity, or lack thereof, of the cholesterol-templated MIPs was developed and involved performing binding studies with four different fluorophores: naphthalene, 2-naphthol, nabumetone, and propranolol HCl. This study also provides, in principle, insight into whether binding sites in the polymers are open. All of the fluorophores are known to form inclusion complexes with free β–CD in water with various binding strengths and are all structurally unrelated to cholesterol. The fluorophores differ in size and cover a range of polarities. The re-binding experiments with the fluorophores were done in a mixed THF and water solvent using 1.16 grams of either MIP or NMIP.

Of the four fluorescent molecules used, naphthalene showed the greatest binding affinity for the cholesterol-templated MIP and NMIP (Table 3.3). After 24 hours a total of 58.4% of naphthalene in the supernatant was bound by MIP and 76.1% by NMIP. Therefore, a molecule structurally unrelated to cholesterol was able to bind significantly to the receptors specific for cholesterol. The MIPs prepared in this study are therefore clearly not specific for cholesterol. The fact that the observed binding of naphthalene to the NMIP is greater than to the MIP for the same guest concentration provides further indications that these MIPs lack specificity.

Naphthalene's ability to bind to the cholesterol-templated MIP and to the NMIP is expected to depend on the size of the molecule, its moderately high binding constant with free β-CD, and the fact that

it is essentially non-polar. Binding affinity is typically described in terms of a binding constant defined as (i.e., for naphthalene (NAP) and β-CD).[50]

$$NAP + CD \leftrightarrow (CD:NAP)$$
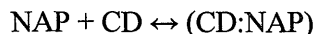
Where (CD:NAP) represents the 1:1 host:guest complex between β-CD and naphthalene. The binding constant for this process, K_{assoc}, is a numerical expression of the binding strength and is $730M^{-1}$.[51] Guests that have high binding constants bind strongly to the receptor and will have a high affinity, while a low binding constant reflects weak binding interactions.[20] K_{assoc} is defined in the conventional manner as,

$$K_{assoc} = [(CD:NAP)]/[CD][NAP]$$

The fact that naphthalene binds more strongly to the NMIP than to the MIP was an unexpected outcome. Given that the MIP is not tailored to bind this particular guest, one would have expected that the ability of the NMIP and MIP to bind naphthalene would be very similar. According to the literature, the cholesterol-templated MIP contains the dimeric form of β-CD as the major product which is linked by the crosslinking agent.[23,44] (See Figure 1.8) On the other hand, the analysis of the NMIP showed a marginal amount of β-CD in its dimeric form and was mostly composed of randomly distributed, crosslinked β-CD monomers.[23,44] The prominent dimeric form in cholesterol-templated MIPs is a result of the pre-organizing effect of the cholesterol template and the resulting end-to-end β-CD cavity formation created which is reported to be on the order of 14-18Å in length.[23,44,45] Naphthalene is $7Å^2$ and cholesterol is 15Å in length.[52] Given that naphthalene is nearly half the size of cholesterol, the molecularly imprinted sites within the MIP are large enough for this guest to diffuse into the polymer and non-specifically bind to those sites by interacting with the functional monomer, β–CD. When such a site is occupied by a naphthalene guest, binding of an additional naphthalene guest to that site will not be independent of the first bound guest, even though the volume available in the molecularly imprinted site is, in principle, large enough to contain two naphthalene molecules. That is, in the MIP not all potential binding sites for naphthalene are independent. In fact, with such a large fraction of the β-CD cavities in

the dimeric form, many of the binding sites will not be independent of previously bound guests. By contrast, the greater degree of binding seen when NMIP was used is consistent with the random monomeric nature of the β–CD molecules within this type of polymer. Since the β–CD molecules in NMIPs are randomly positioned, naphthalene molecules are more likely to come in contact with β–CD molecules and form inclusion complexes leading to the increased percent of binding. Furthermore, each binding event does not reduce the probability of additional binding events.

When 2-naphthol and propranolol HCl were used in binding studies their results were very similar to each other. After four hours 7% of 2-naphthol and 5% of propranolol HCl were bound to the cholesterol-templated MIP and 21% of 2-naphthol and 19.8% of propranolol HCl were bound to the NMIP. These values are substantially smaller than those for naphthalene. 2-Naphthol has a β–CD binding constant of $699M^{-1}$.[53] Propranolol HCl has a binding constant of approximately $10M^{-1}$[48], which is substantially smaller than 2-naphthol's and naphthalene's values. Therefore, the binding constants for each fluorophore with free β-CD seem to have limited value for predicting the extent of binding of the fluorescent guests to the MIP or NMIP. However, it is important to note that the K_{assoc} values available in the literature for the interaction of these fluorophores with free β–CD were all measured in water. The fact that the re-binding measurements in this work were performed in a mixed THF and water solution means the relationship between K_{assoc} trends and re-binding trends should not be over interpreted.

Differences in β-CD binding constants may reflect differences in guest size, hydrogen-bonding ability, and polarity of the guest. If guest binding to the polymers is mediated by CD cavities, some subset of these properties should also play a role in determining the extent of uptake of the guests by the polymers studied here. 2-Naphthol is $7Å$[48] in length, identical to naphthalene. Nabumetone (see below) has a long axis of about $13.4Å$[54] while propranolol HCl is somewhat larger, although we were unable to locate measured or calculated values of its dimensions in the literature. In fact, propranolol HCl is probably closer in size to cholesterol than any of the other fluorophores tested and, as such, might be expected to be a good fit to the molecularly imprinted sites. The fact that its binding to the cholesterol-templated MIP is not that remarkable suggests that simple size consideration does not exert a major impact on the fluorophore binding to the polymers in this study.

Another possible explanation for the decrease in 2-naphthol and propranolol HCl binding to cholesterol-templated MIP and NMIP relative to naphthalene lies with the molecules' polarity. Reported polar surface area values for the three fluorophores are 0, 20.2 and $41.5Å^2$, respectively.[55] Thus, both 2-naphthol and propranolol HCl are significantly more polar than naphthalene and would be expected to

interact less strongly with β-CD cavities. This means the thermodynamic driving force towards binding will be less than in the case with naphthalene, making it more difficult for them to form inclusion complexes with the non-polar inner cavity of β-CD resulting in the reduced binding seen for 2-naphthol and propranolol HCl to MIP and NMIP compared to naphthalene.[56,57] Interestingly, the polar surface area of cholesterol is reported as 20.2Å^2 [55], the same as the 2-naphthol value. The degree of binding of cholesterol to the MIP at 1.16 grams of polymer was 13.3% while that of 2-naphthol was 8.1%. That is to say they are comparable. The same is true for binding of cholesterol and 2-naphthol to the NMIP, where the values are 13.3% and 19.8%, respectively.

Both 2-naphthol and propranolol HCl bind more extensively with the NMIP than with the MIP. We invoke the idea of different binding sites being independent in NMIPs but not in MIPs to explain this difference (see above).

Nabumetone showed the least amount of binding to the cholesterol-templated MIP and NMIP of the four fluorophores; only 4.1% bound to MIP and 3.3% bound to NMIP. The binding constant for nabumetone with free β-CD in water is 2864M^{-1} [58], which is the highest binding constant of the four fluorophores tested. Why, then, does nabumetone show the least amount of binding? Nabumetone is a ketone and the carbonyl oxygen can form hydrogen bonds with the hydroxyl groups that line the outer rims of the β-CD, contributing to the high binding constant with free β-CD. Since the hydroxyl groups on the β-CD have been crosslinked during polymerization in order to form MIPs and NMIPs, they are not available to hydrogen bond with nabumetone's ketone group, thus making it less likely to bind and resulting in the low percentage of binding to the MIPs and NMIPs. However, both 2-naphthol and propranolol HCl can also be involved in hydrogen-bonding to free β-CD, via the hydroxyl group on 2-naphthol and via the hydroxyl and amino groups on propranolol HCl. Hydrogen bonding interactions of hydroxyl and amino groups with the hydroxyl groups of β-CD cavities should be stronger than those between a ketone and the β-CD hydroxyls. Therefore, if elimination of hydrogen bonding were a key factor controlling binding to the polymers, one would expect a dramatic reduction in 2-naphthol and propranolol HCl binding to polymers compared to binding to free β-CD as well and that the change would be greater than in the case of the more weakly hydrogen bonded nabumetone.

The polar surface area of nabumetone is reported to be 26.3 Å^2, similar to that of 2-naphthol.[55] Based on this parameter, one would expect nabumetone's binding to the MIP and NMIP to be comparable to that of 2-naphthol, while in fact it is weaker. In terms of dimensions, nabumetone's length in its extended conformation (13.4Å) approached that of cholesterol (15Å) and its binding to both polymers is

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poor, as is cholesterol's. One might invoke a size threshold below which binding to the polymers is reasonably strong (i.e., naphthalene and 2-naphthol), but above which binding is weaker (i.e., nabumetone and cholesterol). However, propranolol HCl is longer, in its extended configuration, than nabumetone but its binding to the polymers is stronger. So again, a simple size analysis does not fully explain the observed behaviour. On the other hand, propranolol HCl is much more polar than nabumetone. Although it is speculative, we propose that binding to the polymers is controlled by a combination of size and polarity with small, low polarity guests being more strongly bound than larger, more polar guests.

Although our analysis of which factors are controlling guest binding to the cholesterol-templated MIP and NMIP must remain speculative, the results clearly show that the receptors within this particular MIP are not specific in nature to their template.

Selectivity is another advantage often attributed to the molecular imprinting technique, and it was important to examine whether or not the cholesterol-templated MIP would selectively bind its template over a competing molecule. It was also important to develop an indirect way to measure cholesterol re-binding using luminescence spectroscopy in order to confirm the results of the cholesterol re-binding experiments that were analysed using GC. With these goals in mind competitive binding studies were carried out. Competitive binding experiments were performed with naphthalene and 2-naphthol as the competitors. These two fluorophores were used because they showed the greatest degree of binding to the cholesterol-imprinted MIP and NMIP from the previous binding studies (Table 3.3). The competitive binding experiments would elucidate whether any cholesterol was in fact re-binding to its tailor made receptors and as a result blocking the fluorophore from interacting with the sites within the polymer. If this happens, one expects to observe greater fluorescence intensity in the supernatant compared to the cholesterol-free condition.

When competitive binding experiments were carried out using naphthalene as the competitor, its fluorescence intensity in the supernatant decreased over time (Figure 3.11). There is a larger decrease in fluorescence intensity in the supernatant when NMIP was used compared to MIP. However, there is a small decrease in the amount of fluorophore bound to MIP and NMIP when in competition with cholesterol. Table 3.4 shows that naphthalene binds to MIP 12% less when cholesterol is present compared to when it is absent; when cholesterol was not present, 58.4% of naphthalene was bound by MIP. Only 46.8% of naphthalene was bound by MIP in the presence of cholesterol. Therefore, the cholesterol present during competitive binding experiments has, at most, a small inhibitory effect on naphthalene's ability to bind to the polymer. Given that the difference between the amounts of fluorophore bound to MIP when cholesterol is present and absent is small, it is reasonable to state that the

cholesterol-templated MIP does not prefer its template over the fluorophore, supporting the notion that the MIPs lack selectivity.

A similar result was observed when 2-naphthol was used as the competitor. Approximately 2% less 2-naphthol was bound to the MIP when cholesterol was present compared to when it was absent. When cholesterol was present during the competitive binding with NMIP only about 3% less 2-naphthol was able to bind to the polymer compared to when cholesterol was absent. Overall the competitive binding experiments indicate that the MIPs made are not selectively binding their template when placed in a mixture of molecules that include the template.

Another approach to assess the selectivity of the cholesterol-templated MIP entailed binding naphthalene to the cholesterol-templated MIP followed by the addition of cholesterol in order to see if the template would displace the non-specific guest from the binding sites. If this happened there would be an increase in the fluorescence intensity within the supernatant subsequent to cholesterol addition. Figure 3.13 illustrates the results of this experiment. Within the first 24 hours, there is a decrease in the naphthalene fluorescence intensity in the supernatant when MIP and NMIP were used. These results are consistent with those shown in Figure 3.7, thus naphthalene is bound to the MIP and NMIP. When cholesterol was added at the 24th hour, there was very little change in the fluorescence intensity of the supernatant. Moreover, there is no change in the fluorescence intensity in the supernatant after the addition of cholesterol between hours 24 and 48. Due to the insignificant change in the fluorescence intensity in the supernatant after the template was added to the binding mixture, the results indicate no displacement of the fluorophore by cholesterol, therefore providing additional evidence that the cholesterol-templated MIP is not selective towards its template.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

The outcome of this study consistently suggests that cholesterol-templated molecularly imprinted polymers made with β-CD are neither specific nor selective for their template molecule. The results presented in this paper do not coincide with the literature although the synthesis and re-binding procedures carried out were identical.

The cholesterol-templated MIPs did not demonstrate an inclination to specifically re-bind to their template. These results were consistent when re-binding studies were carried out in various solvent systems, such as THF and THF/water. The trivial difference between the MIP's and NMIP's abilities to bind cholesterol contradicts the notion that molecular imprinting provides a highly specific product with the ability to demonstrate molecular recognition behaviour. In fact, there were some instances where the MIP did not bind the template at all within the re-binding environment. Furthermore, there was no difference in the amount of cholesterol uptake by MIP or NMIP when a greater quantity of the polymer was used during re-binding.

Verification for the MIP's lack of specificity for cholesterol was provided when structurally unrelated molecules (the fluorophores) were used as guests in binding experiments with cholesterol-templated MIPs and NMIPs. The four fluorophores used showed a range of binding affinities to the cholesterol-templated MIP and NMIP. Furthermore, the degree to which each fluorophore bound to both types of polymers seems to depend on their individual polarities and possibly their sizes. Naphthalene was more effectively bound to the polymers than the other fluorophores used. Naphthalene is the least polar and one of the smallest of the fluorophores used here.

Competitive re-binding studies done with cholesterol, naphthalene, and 2-naphthol indicated the MIP's lack of selectivity for its template. The results suggested that both naphthalene and 2-naphthol inhibited cholesterol from binding to its MIP and the NMIP. In addition, when displacement studies were performed, the template failed to displace the non-specific fluorophore from the binding sites in the cholesterol-templated MIP and the NMIP.

Lastly, although the results are contrary to the literature they consistently suggest that the cholesterol-templated MIPs presently synthesized are not as specific or selective for the template as originally hoped. There is very little difference between the degree of template binding seen between the MIP and NMIP, along with unrelated molecules being able to non-specifically bind to both polymers. In addition, the cholesterol-templated MIP does not exhibit any selectivity towards its template over unrelated molecules. These outcomes make it unlikely that an effective MIP system based on CDs as the functional monomer can be designed to trap traces of steroid-like endocrine disruptors in water sources.

With respect to any future work using this system of polymers there are a couple of options that should be considered. The most important would be addressing the issue of improving the specificity and selectivity of these polymers toward their template. One potential way to achieve this would be to introduce another functional monomer or choose a completely different one and omit the use of β-CDs altogether. However, when choosing a different functional monomer a number of properties would have to be considered such as its binding constant and polarity. The binding constant between the template and functional monomer would have to be very high and the polarities of both should be similar in order to ensure a highly specific and selective interaction. Secondly, increasing the amount of crosslinker during polymer synthesis may contribute to a favourable outcome in this polymer system. By adding more crosslinker the product would become more rigid, thus producing a tighter imprinted site and potentially improving the imprinted sites' specificity for its template.

Another approach to consider would be a method to separate the dimeric forms of the polymers from the trimers and monomers that are simultaneously synthesized during the molecular imprinting of cholesterol. Since the product is a mixture containing a small percentage of monomers and trimers in addition to the predominant dimers, it would be useful to merely collect the dimers. Ultimately, this would allow for only the optimal dimeric form of the MIPs to be used in subsequent binding experiments.

Another issue that should be addressed is a way to maximize re-binding of the template to the polymer by performing re-binding experiments within the best solvent for this particular system. Since cholesterol and β-CD have a very high binding constant in water $(17000M^{-1})$[52] it would be interesting to see if performing the re-binding studies in water would alter the extent of template binding.

APPENDIX A

CALIBRATION CURVES FOR CHOLESTEROL

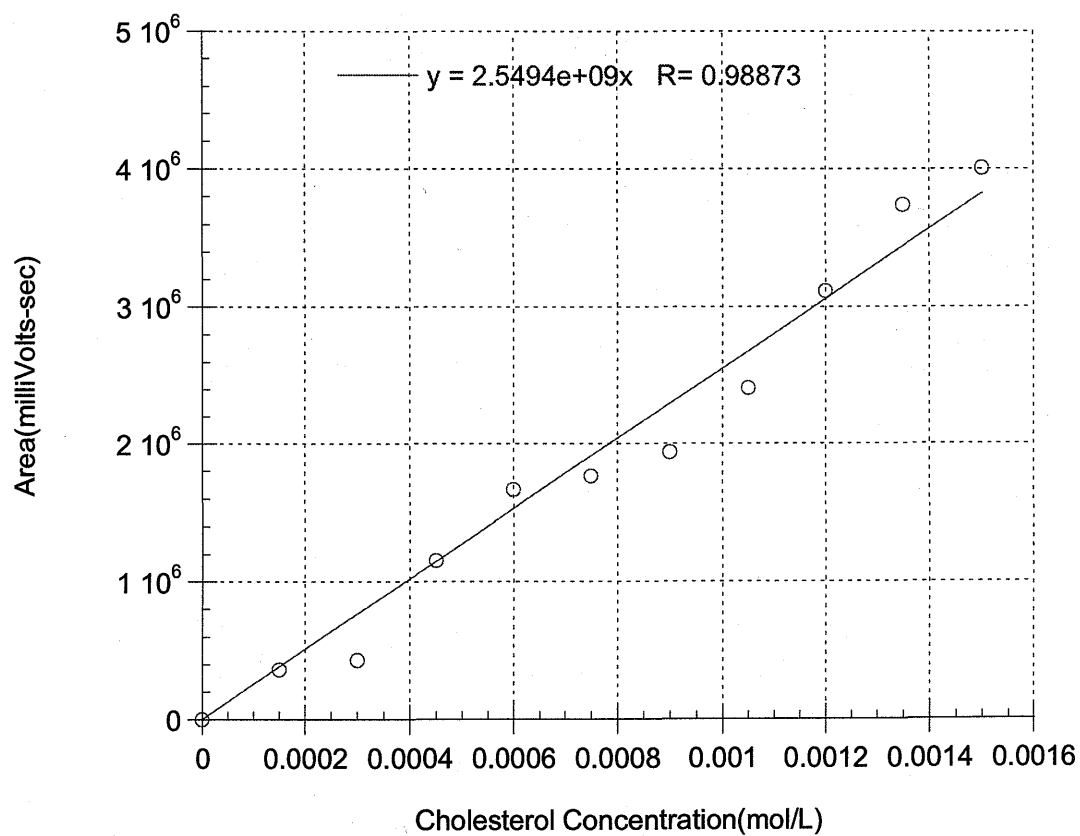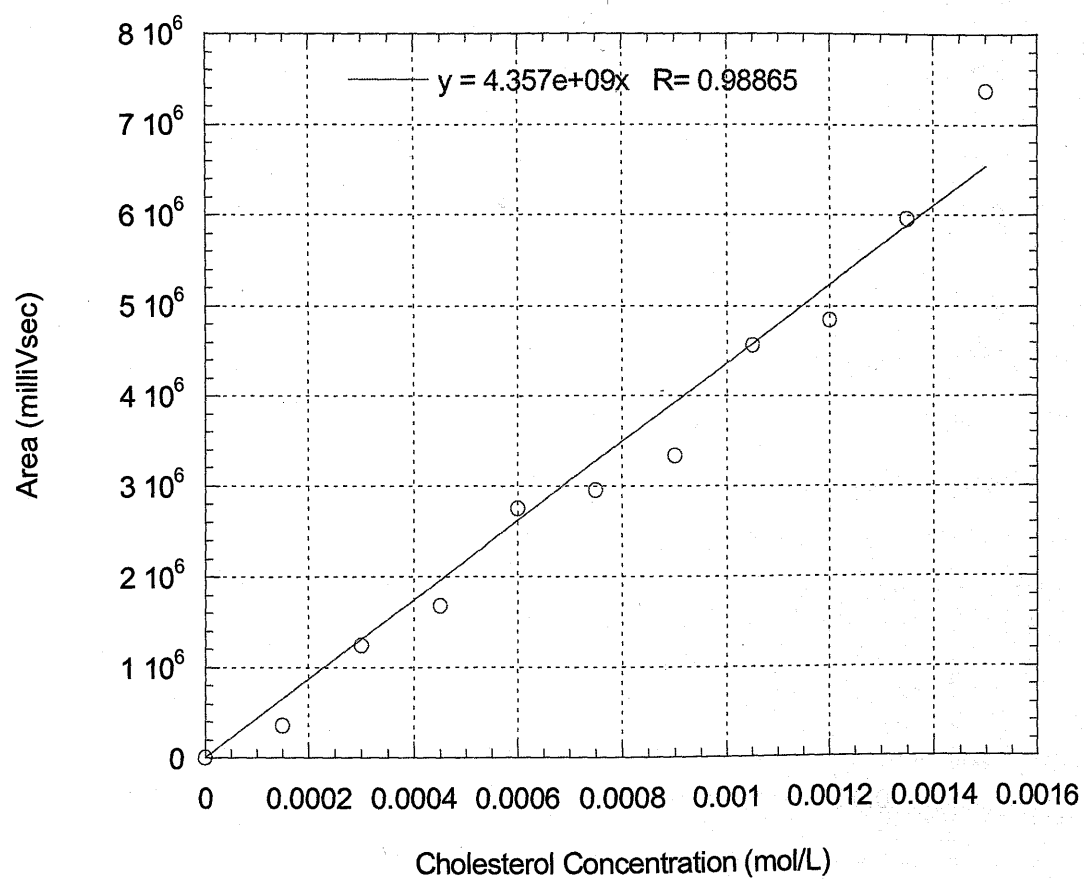
Figure A.1 Calibration Curve for Cholesterol in THF

Figure A.2 Calibration Curve for Cholesterol in THF and Water Solution.

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